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# IV INTERNATIONAL WORKSHOP ON MEMBRANE BIOTECHNOLOGY AND MEMBRANE BIOMATERIALS

29 May - 2 June, 1991

La Manga, Murcia, Spain



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Este Libro de Resúmenes ha sido editado por Juan C. Gómez Fernández y Francisco J. Aranda Martínez, Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia.

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# PROGRAMME

Wednesday 29th May

From 12:00 on Checking-in, Hotel Galua, La Manga
20:00 Dinner
22:00 Get together party

Thursday 30th May

BASIC STUDIES ON BIOMEMBRANES

*Chairman:* C. Gutierrez Merino

9:00 - 9:35 "Basic and Technological Studies of Biomembrane Component", Dennis Chapman (AL1)

9:35 - 10:10 "Translocation of Proteins in *Escherichia coli*", Antoinette Killian (AL2)

10:10 - 10:45 "Dynamics X-ray Diffraction Studies of Phase Transitions in Lipid-water systems", Peter J. Quinn (AL3)

10:45 - 11:15 Coffee

*Chairman:* K. Wirtz

11:15 - 11:50 "Platelet Aggregating Factor (PAF), a Lipidic mediator with a Structural Activity in Bilayers: a Model sustaining a Biological relevance for Asymmetric phospholipids", Claude Wolf (AL4)

11:50 - 12:25 "Localization and Dynamics of α-Tocopherol in Membranes", Juan C. Gómez Fernández (AL5)

12:25 - 13:00 "Membrane Stabilization by Antioxidant Recycling Mechanisms: the Vitamin E Cycle and the Synergistic Action of the Dihydrolipoic Acid/Thioctic Acid Redox Couple", Lester Packer (AL6)

13:00 - 13:15 WELCOME TO THE MEETING
13:15 Lunch

*Chairman:* M. Cortijo

16:00 - 16:35 "Fluorescent Phospholipids in Studies on Lipid-Protein Interactions", Karel W.A. Wirtz (AL7)

16:35 - 17:10 "Phase Behaviour of Phosphatidic Acids Thermodynamic Stability of Phosphatidic Acid Vesicles", H. Hauser (AL8)

17:10 - 17:45 "Consensus Modeling of Lipid Bilayers", Bruce P. Gaber (AL9)

17:45 - 19:30 Posters session and coffee

20:00 Dinner

Friday 31st. May

MEMBRANE FUSION AND LIPOSOMES

*Chairman:* D. Chapman

9:00 - 9:35 "Amphiphiles and Membrane Fusion", Felix M. Goñi (AL10)

9:35 - 10:10 "Membrane Fusion Activity of Influenza Virus and reconstituted Influenza Virus Envelopes (Virosomes)", Jan Wilschut (AL11)

10:10 - 10:45 "Full Length Recombinant CD4 Electroinserted in the Red Blood Cell membrane as a Long-Lived Potential Therapeutic agent against AIDS", Claude Nicolau (AL12)

10:45 - 11:15 Coffee

*Chairman:* D.W. Grainger

11:15 - 11:50 "Liposomes as Carriers of Antigens and Adjuvants" Carl R. Alving (AL13)

11:50 - 12:25 "Liposomes for Cell Targeting: Current Status and Prospects", Lee Leserman (AL14)

12:25 - 13:00 "Treatment of Tumors Using Cytostatic-Containing (Immuno)Liposomes", G. Storm (AL15)

13:15 Lunch

A visit to Murcia will be organized for this afternoon.

Saturday 1st. June

MEMBRANE MIMICRY AND BIOMATERIALS

*Chairman:* L. Packer

9:00 - 9:35 "Binding, Interaction, and Organization of Protein with Lipid Model Membranes", David W. Grainger (AL16)

9:35 - 10:10 "Equilibrium, Transport and Kinetics of Antibodies on Planar Model Membranes Measured by Quantitative Fluorescence Microscopy", Nancy L. Thompson (AL17)

10:10 - 10:45 "Lipid Matrices and Receptor-Ligand Interactions", G. Graham Shipley (AL18)

10:45 - 11:15 Coffee

*Chairman:* Félix M. Goñi

11:15 - 11:50 "Role of Membrane Components on Anthracycline Cytotoxicity", José M. González-Ros (AL19)

11:50 - 12:25 "Polymeric Drug-Carriers: Haematocompatibility", Ruth Duncan (AL20)

12:25 - 13:00 "Liposome Encapsulated Hemoglobin; *in vivo* Efficacy of a Synthetic Red Cell Substitute", Alan Rudolph (AL21)

13:15 Lunch

*Chairman:* B.P. Gaber

16:00 - 16:35 "Polihydroxialkanoates, a Family of Biodegradable Plastics from Bacteria", F. Rodríguez Valera (AL22)

16:35 - 17:10 "Positive Staining of Outer Membrane Lipopolysaccharides of Gram Negative Bacteria: Image Analysis for Membrane Biodiagnostics", Jacobs S. Hanker (AL23)

17:10 - 17:30 Coffee

*Chairman:* C. Nicolau

17:30 - 18:05 "Hydrophobic Pulmonary Surfactant Proteins in Model Lipid Systems", Kevin M.W. Keough (AL24)

18:05 - 18:40 "Axon, Schwann Cell, and Collagen Fibril Interactions in Avulsive Peripheral Nerve Injury Repair with a Biodegradable neuroprosthesis and a Basement Membrane gel", B.L. Giammara (AL25)

18:40 - 19:15 Summing up and prospects in the field

20:00 Meeting dinner

Sunday 2nd, June

Checking out after breakfast and not later than 11:00 am

# ABSTRACTS LECTURES

# BASIC & TECHNOLOGICAL STUDIES OF BIOMEMBRANE COMPONENTS

Dennis Chapman, FRS, Department of Protein & Molecular Biology
Royal Free Hospital School of Medicine, Rowland Hill Street,
London.

This talk describes recent basic studies of polypeptides and proteins using FTIR spectroscopic techniques e.g. signal polypeptides, water soluble and membrane proteins. The qualitative and quantitative information which can be deduced concerning secondary structure will be shown. The possibility of distinguishing a normal α-helical structure from a $3_{10}$ helical structure by this technique will be indicated.

Recent technological studies will be described of blood coagulation behaviour in the presence of various lipid polar surfaces including negative charges, positive charges and Zwitter-ion charges. The effect of these surfaces in blood has been examined using thrombelastography and blood factor analysis. Potential technological applications will be indicated.

Translocation of proteins in *Escherichia coli*

J.A. Killian and B. de Kruijff, Centre for Biomembranes and Lipid Enzymology,
University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

Recently much attention has been focussed upon elucidating the mechanism of protein export out of the cytoplasm of bacterial cells. In particular intriguing is the question of how these often hydrophilic proteins are able to cross the plasma membrane. Of key importance for this process is the presence of a functional signal sequence. Proteins that are destined for export are synthesized as precursors with a 20-25 amino acid long temporal N-terminal extension, the signal sequence, that consists of a positively charged N-terminus, followed by a stretch of 10-15 hydrophobic amino acids and a slightly more polar C-terminal region, containing the cleavage site.

The translocation process can de divided into several steps: [1] Efficient targeting of the precursor protein to the membrane, [2] the actual translocation step in which the protein crosses the membrane and [3] cleavage of the signal sequence by the enzyme leader peptidase, which has its active site at the periplasmic site of the membrane. A number of proteins, all products of the *sec*-genes, are known to be involved in various stages of the translocation process. These and other factors, such as the requirement for ATP and a membrane potential, will be briefly discussed. The central question however that will be addressed is the role of lipids and signal-sequence/lipid interactions in protein translocation (recently reviewed in ref. 1).

It has been unambiguously demonstrated that the presence of negatively charged lipids in the target membrane is required for optimal translocation (1,2). One possibility is that these lipids promote efficient binding of the precursor protein to the membrane by electrostatic interactions with the positively charged N-terminus of the signal sequence. In addition these lipids could induce conformational changes in the signal sequence that are important for translocation (3-5). Hydrophobicity is a hallmark of functional signal sequences and could be essential for subsequent insertion of the protein into the membrane. Finally, it has been proposed that the interaction of the signal sequence with the membrane lipids may lead to the formation of transient non-bilayer structures, resulting in the formation of a lipidic pore through which the protein may cross the membrane (6).

References:

(1) G. J. de Vrije et al. (1990) Mol. Microbiol. 4, 143-150
(2) R. Kusters et al. (1991) J. Biol. Chem., in press
(3) A.M. Batenburg et al., (1988) J. Biol. Chem. 263, 4202-4207
(4) R.A. Demel et al. (1990) Biochim. Biophys. Acta 1027, 155-162
(5) J.A. Killian et al. (1990) Biochemistry 29, 8131-8137
(6) J.A. Killian et al. (1990) EMBO J. 9, 815-819

# Dynamic X-ray Diffraction Studies of Phase Transitions in Lipid-water Systems

P J Quinn

Department of Biochemistry, King's College London, Campden Hill, London W8 7AH, UK

Membrane lipids when dispersed in aqueous systems display complex polymorphic behaviour as a function of temperature and hydration. The usual method of characterizing this behaviour is to determine the temperature and endotherms of the various transitions that take place between the various phases and then to assign
structural parameters to each phase on either side of the transition using X-ray difffraction, nuclear magnetic resonance spectroscopy, vibrational spectroscopy etc. The thermal data is obtained by temperature scanning often at rates of 1°/min or faster and it is not always the case that the equilibrium phase state is achieved. Conventional methods for structural assignments are essentially static and the sample can be examined under equilibrium conditions.

In order to reconcile the thermal and structural parameters associated with polymorphic phase behaviour X-ray diffraction studies have been performed under identical conditions used to provide thermal data. The high X-ray flux required to record diffraction patterns on the time-scale of ms has been achieved using synchrotron radiation at the Daresbury (UK) Laboratory. Studies of the mechanism and kinetics of phase transitions in representative lipid-water systems will be described.

Ref. Tenchov, B. G. and Quinn, P. J. (1989) Two-state and continuous phase transitions in lipid bilayers; a time-resolved X-ray diffraction study. Liq. Cryst., 6, 1691-1695.

Lis, L. J. and Quinn, P. J. (1991) The application of synchrotron X-radiation for the study of phase transitions in lipid model membrane systems. Acta Cryst, 24, 48-60.

# PLATELET AGREGATING FACTOR (PAF), A LIPIDIC MEDIATOR WITH A STRUCTURAL ACTIVITY IN BILAYERS: A MODEL SUSTAINING A BIOLOGICAL RELEVANCE FOR ASYMETRIC PHOSPHOLIPIDS.

WOLF Claude, URA CNRS 1283 CHU St. Antoine, 27 Rue Chaligny, Paris 75551 Cedex 12.

PAF and other inactive highly asymmetric phospholipids (lysoPAF, lysoPC) share an identical propensity to segregate from the surrounding matrix phospholipids (ovolecithin, DMPC) to constitute interdigitated lamella with a "disorienting" effect on the local director of the plane lamellar phase.

The observation is made after various techniques: oriented multibilayers probed with 5NS studied by ESR, oriented multibilayers studied by $^{31}P$ NMR, vesicles studied with small angle X Ray diffraction.

The occurence of thin walled blisters made of segregated PAF interrupting the bilayer is proposed as a possible arrangement for the asymmetric phospholipid.

The antagonistic actions of increasing the temperature (monitored by temperature-time resolved X-ray diffractograms) and of cholesterol are described. The lifetime of the membrane structural alteration brought about by the asymmetric phospholipid is discussed accordingly. Preliminary data (X ray, DSC) suggest that low levels of diglycerides (5%) act as an enhancer for the membrane perturbation.

Finally, the *in vivo* activity of asymmetric molecular species of phospholipids (naturally constitutive in biomembrane) is questioned after a detailled DCI-Mass Spectrometry analysis of the diglycerides released by thrombin-activated rat platelets. The enrichment (relative to the possible precursor phospholipids) of diglycerides in asymmetric molecular species suggests that PLase C could act preferentially on phospholipids with inequivalent acyl chains. This suggests, in turn, that the structural perturbation brought by asymmetric phospholipids in the platelet membrane is sensed by the activated enzymes during the thrombin-induced agregation.

# LOCALIZATION AND DYNAMICS OF α-TOCOPHEROL IN MEMBRANES

J.C.Gómez-Fernández, F.J.Aranda and J. Villalaín
Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, E-30071 MURCIA, SPAIN

α-Tocopherol has been studied in model membrane systems in order to investigate its mode of interaction with phospholipids and its dynamics in these membranes.

We have shown that α-tocopherol may affect the phase transition of DPPC (1), having a clear preference to partition in the most fluid domains (2). On the other hand α-tocopherol has a high diffusion coefficient comparable to phospholipids (3). α-Tocopherol is able of modulating the lipid polymorphism properties of phosphatidylethanolamines, and the $H_{II}$ phase is stabilized in its presence (4). Finally, the location of α-tocopherol in the membrane bilayer has been studied, showing that the chromanol moiety of the molecule is located near the lipid-water interphase of the membrane (3).

1. Villalaín, J., Aranda, F.J. and Gómez-Fernández, J.C. (1987) Eur. J. Biochem. 158, 141.147
2. Ortiz, A., Aranda, F.J. and Gómez-Fernández, J.C. (1987) Biochim. Biophys. Acta. 989, 214-222
3. Aranda, F.J. et al. (1989) Biochim. Biophys. Acta 985, 26-32
4. Micol, V., Aranda, F.J., Villalaín, J. and Gómez-Fernández, J.C. (1990) Biochim. Biophys. Acta 1022, 194-202-202

This work has been supported in part by grant no. PM91/0044 from DGICYT (SPAIN).

# "Membrane Stabilization by Antioxidant Recycling Mechanisms: the Vitamin E Cycle and the Synergistic Action of the Dihydrolipoic Acid/Thioctic Acid Redox Couple"

by Dr. Lester Packer

Department of Molecular and Cell Biology, 251 Life Sciences Addition,University of California, Berkeley, CA 94720,USA,Tel: (415) 642-1872,FAX: (415) 642-8313

Living cells have three main systems for protection and repair under oxidative stress: (1) direct antioxidant enzymes (SOD, catalase, peroxidases), (2) proteases and phospholipases activated by oxidative modification of membranes, and (3) lipid- and water-soluble antioxidants. (1) and (2) constitute the two lines of enzymic antioxidant defense, while (3) has been considered in the past to be mainly non-enzymic and dependent on nutritional factors. Our recent studies show that this is not the case: both non-enzymatic and enzymatic exist and knowledge of these mechanisms can be exploited to stabilize membranes.

Vitamin E is believed to be the major lipid-soluble chain-breaking antioxidant in membranes. However its membrane concentration is usually lower than 0.5 - 0.1 nmoles/mg of protein or 0.05 - 0.1 mole% of membrane phospholipids. The rates of lipid radical generation dependent on electron transport (e.g. NADPH-dependent lipid peroxidation in liver microsomes) may be as high as 1 - 5 nmoles/mg of protein per minute. Nevertheless, under physiological conditions the low concentration of vitamin E is sufficient to prevent membrane oxidative damage. With Dr. Valerian Kagan, we have demonstrated that vitamin E can be enzymically regenerated from its chromanoxyl radicals formed in the course of peroxidation. ESR and HPLC data show that chromanoxyl radicals of vitamin E can be reduced by NADPH-, NADH-, and succinate-dependent electron transporting enzymes of microsomes and mitochondria. Ubiquinones act as co-factors in enzymic regeneration of vitamin E. These enzymes involved in the regeneration of lipid-soluble phenolic antioxidants constitute the third line of antioxidant defense, controlled by the cell genome.

Ascorbate, glutathione, dihydrolipoate, and other reductants can synergistically enhance vitamin E regeneration. The well known synergistic effects of these physiologically important antioxidants (reductants) with vitamin E are mediated via their ability to transfer electrons necessary for recycling chromanoxyl radicals in membranes. Thus, vitamin E molecules (tocopherols and tocotrienols) possess a unique ability to act as membrane free radical harvesting centers due to their enzymic and non-enzymic regeneration in membranes. In this regard, α-tocotrienol (having an unsaturated isoprenoid side chain) as compared to α-tocopherol has been found to possess 40 - 60X greater antioxidant potency. Three factors seem to explain this remarkable effect: greater recycling activity, more homogenous membrane distribution, and greater membrane mobility.

The thioctic acid/dihydrolipoic acid couple (TA/DHLA) is a unique system. Normally lipoamide exists as the co-factor of α-keto-dehydrogenases and is covalently bound in animals. Thus, its presence is at the level of micronutrient. However, TA, which can readily be fed to animals, has been found to confer protection in tissues and membranes against oxidative damage. This is believed to occur because TA after it is absorbed, is reduced enzymatically or non-enzymatically and then becomes active as an antioxidant. It acts a a "double-edged sword" in that it appears to interact directly with the membrane to reduce tocopheroxyl radicals (weak effect) or to reduce ascorbate which in turn acts at membranes to reduce tocopheroxyl radicals (stronger effect). This unique mechanism of the TA/DHLA couple works in recycling of vitamin E both in membranes and in low density lipoproteins (LDL) where it acts to stabilize them. A redox cycle illustrating the interactions between these antioxidants in membranes and LDL in their stabilization will be presented.

## Fluorescent Phospholipids in Studies on Lipid-Protein Interactions

K.W.A. Wirtz, Centre for Biomembranes and Lipid Enzymology, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

Phospholipids that carry a parinaroyl (C18:4) (Par) chain or a pyrene-labeled fatty acyl (PyrCx) chain, have been used to study the lipid binding and transfer properties of phospholipid transfer proteins (Berkhout et al., Biochemistry 23(1984)1505; Somerharju et al., Biochemistry 26(1987)7193; Van Paridon et al., BBA 898(1987)172; Van Paridon et al., Biochemistry 27(1988)6208). Incorporation of these phospholipids from vesicles into transfer proteins can be measured directly as these lipids bound to proteins have fluorescent properties different from those in vesicles. Time-resolved fluorescence measurements of phosphatidylcholine (PC) transfer protein carrying one molecule of 1-Par-PC or 2-Par-PC, have indicated that each Par chain has its own binding site and that the 1- and 2-Par chains accommodated in the protein make an estimated angle of 60-90° with one another. By measuring the binding of PC analogs carrying PyrCx chains of different length (Cx is 6-14 C-atoms) at either the 1- or 2-position, it has been demonstrated that the 1-acyl and 2-acyl binding sites have completely different properties and that, as a result, the PC-transfer protein can discriminate between positional isomers of PC.

Methods have been developed to synthesize 2-PyrCx phosphatidylinositol (Pyr-PI) and the phosphorylated derivatives Pyr-PI-4-P (Pyr-PIP) and Pyr-PI-4,5-$P_2$ (Pyr-$PIP_2$) (Gadella et al., Biochemistry 29(1990)3389). The behavior of these PI derivatives in model membranes was investigated by use of the pyrene excimer/monomer (E/M) fluorescence technique. From changes in E/M it could be inferred that the collision frequency in PC bilayers decreased in the order PI > PIP > $PIP_2$ which reflects the increase in negative charge on the polar head group. Experiments in which $Ca^{2+}$-ATPase is reconstituted in a PC-bilayer containing these fluorescent PI derivatives, will be discussed (Verbist et al. BBA (1991) in press).

# Phase behaviour of phosphatidic acids: thermodynamic stability of phosphatidic acid vesicles.

H. Hauser and C.-C. Yin.
Laboratorium für Biochemie, Eidgenössische Technische Hochschule, ETH Zentrum, CH 8092 Zürich, Switzerland.

ABSTRACT

Phosphatidic acids at neutral pH form smectic (lamellar) phases as evident from X-ray diffraction. Subjecting phosphatidic acid dispersions to sonication or alternatively to a pH-gradient by rapidly raising the pH of the external medium to 10-12 produces small unilamellar vesicles of diameter < 50 nm. With both methods the phosphatidic acid dispersion is energized, i.e., the formation of small unilamellar vesicles requires the input of external energy in one form or another. By implication, the resulting dispersion of small unilamellar vesicles is thermodynamically unstable. This is demonstrated by permeability measurements based on fluorescence spectroscopy, gel filtration on calibrated Sepharose CL-4B, freeze-fracture electron microscopy and $^{31}P$ NMR measurements. Indeed, as predicted the small unilamellar vesicles of phosphatidate are unstable in the absence of a pH gradient. The pH gradient used to generate the small unilamellar vesicles stabilizes these vesicles. On the other hand, a reverse pH gradient applied to phosphatidate vesicles such that the external medium is acidic and the vesicle cavity remains neutral, destabilizes the phospholipid bilayer leading to vesicle aggregation and fusion. This process is accompanied by leakage of vesicle contents.

# Consensus Modeling of Lipid Bilayers

Bruce Paul Gaber

Center for Bio/Molecular Science and Engineering
Naval Research Laboratory
Washington, DC 20375

Over the past several years our efforts to develop molecular graphics depictions of lipid microstructures have focused on the representation of the lipid bilayer as it exists in the crystalline state (1). However reliance on crystallographic data for development of lipid models places serious limits on the modeler. From a practical point of view, there simply never will be the number of structures solved for lipids as there are for proteins. Lipids are intrinsically difficult to crytallize. Further, nothing comparable to the burgeoning field of protein engineering provides the incentive for extensive lipid crystallography. More fundamentally, however, we must remember the unique dynamic properties of lipids. Crystal structures of lipids give a static, highly organized view of the bilayer. This view, while useful in understanding some aspects of lipid structure, may deceive us badly if we believe that it represents the nature of lipids in their fluid-like, dynamic natural environment. One solution to this dilemma is the recognition that there exists a vast body of biophysical data from which a considerable amount of structural information can be extracted and used for construction of lipid models. I have called this approach "consensus modeling" since the idea is to develop a model which represents a concensus of the available experimental data, with most emphasis placed on that data regarded by the experimentalist as most accurate and reliable. This approach will be demonstrated by the construction of a model, based upon published X-ray and neutron diffraction studies, of the interaction of dioleoyl phosphatidylcholine with water and a hydrophobic dipeptide.

1) B.P. Gaber, M. Nagumo, W.R. Light, I. Chandrasekhar, and N. Pattabiraman "Molecular Modeling of the Phospholipid Bilayer", in **Technological Applications of Lipid Microstructures**, B.P. Gaber, J.M. Schnur and D. Chapman, eds., Plenum Publishing Corporation, New York (1988).

# THE INVOLVEMENT OF NON-LAMELLAR PHASES IN PHOSPHOLIPASE C-INDUCED LIPOSOME FUSION.

José-Luis Nieva, Alicia Alonso and Félix M. Goñi

Department of Biochemistry, University of the Basque Country, P.O. Box 644, 48080 Bilbao, Spain.

Previous work from this laboratory has shown that phospholipase C induces non-leaky fusion of large and small unilamellar liposomes, presumably via diacylglycerol formation (Nieva et al., Biochemistry 28, 7364-7367, 1989). Further studies on the same system have made use of fast freeze and conventional freeze fracture electron microscopy, $^{31}$P-NMR and X-ray diffraction. Electron microscopy reveals that phospholipase C-activity induces massive aggregation and fusion of large unilamellar vesicles, and leads to the formation of a "sealed" lipid aggregate; in some areas lipid arranges into a honeycomb structure which is probably a precursor of a discontinuous inverted cubic phase. $^{31}$P-NMR spectra of a PC/PE/CHOL dispersion containing 5% diacylglycerol show a thermotropic transition from a lamellar to an isotropic phase at about 60ºC; such transition is not observed in the absence of diacylglycerol. Finally, X-ray diffraction indicates the presence of cubic phases in diacylglycerol-containing lipid dispersions. These data are interpreted in terms of a model for phospholipase C-induced membrane fusion.

# MEMBRANE FUSION ACTIVITY OF INFLUENZA VIRUS AND RECONSTITUTED INFLUENZA VIRUS ENVELOPES (VIROSOMES)

J. Wilschut[1], R. Bron[1], J. Dijkstra[1], L.C. van Ginkel[1], A. Ortiz[1,¶], J.D. Lear[2], M. Rafalski[2] and W.F. DeGrado[2]
[1]Lab. of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands;
[2]DuPont-Merck Pharmaceuticals Co., P.O. Box 80328, Wilmington, DE 19880-0328, USA;
[¶]Present address: Dept. of Biochemistry and Mol. Biology, Veterinary Faculty, University of Murcia, Murcia, Spain.

Influenza virus enters its host cell by receptor-mediated endocytosis, routing the virus particles, intact, to the endosomal cell compartment. Within the endosomes, the lumenal mildly acidic pH triggers a conformational change in the major viral spike glycoprotein, the hemagglutinin (HA), resulting in exposure of the hydrophobic N-terminus of the HA2 subunit. This hydrophobic "fusion segment" is thought to penetrate into the endosomal membrane, resulting in fusion of the viral envelope with the endosomal membrane and release of the viral nucleocapsid into the cellular cytosol. Using kinetic fluorescence assays based on lipid mixing, we have characterized the pH-dependent fusion activity of intact influenza virus in various model systems, involving both artificial and biological target membranes. Also the physiological intracellular fusion process can be monitored using fluorescently labeled virus.

At present, we are focussing on two specific aspects of the membrane fusion activity of influenza virus. First, the interaction with lipid bilayer vesicles of synthetic peptides corresponding to the fusion sequences of fusion-active HA and mutant HA's with altered fusion activity (Gething et al., J. Cell Biol. 102, 1986, 11). A distinct correlation between the fusion activity of a particular HA and the membrane-penetrating capacity of the corresponding fusion peptide could be demonstrated. Membrane-penetrating capacity of the peptides appears to be governed by their (in)ability to adopt an α-helical conformation upon interaction with lipids. Second, we are investigating reconstituted viral envelopes (virosomes), both as a model for the native virus in cellular entry studies and as a carrier system to introduce biologically active molecules into cells. Influenza virosomes efficiently deliver encapsulated substances to the cellular cytosol after receptor-mediated endocytosis. Delivery of toxin molecules, such as the Diphteria toxin A chain, allows a quantitative assessment of the intracellular fusion process. Virosomes, in a study of the mechanism of action of bacterial lipopolysaccharide (LPS), have also been used to introduce this immunomodulator into membranes of murine B lymphocytes.

# FULL LENGTH RECOMBINANT CD4 ELECTROINSERTED IN THE RED BLOOD CELL MEMBRANE AS A LONG-LIVED POTENTIAL THERAPEUTIC AGENT AGAINST AIDS

Claude Nicolau, Center for Blood Research, Harvard Medical School, Boston, MA 02611, USA

The strong affinity of the CD4 receptor for the HIV envelope glycoprotein gp120 has suggested some strategies for its use as therapeutic agent against AIDS. Despite CD4's efficiency in blocking HIV infection *in vitro* and some success in SIV-infected M.rhesus, it appeared that very high amount of soluble CD4 may be required *in vivo* to achieve "therapeutic concentrations", largely due to the short half life time of sCD4 and even of IgG-CD4 constructs. There might be significant advantages in having a long-lived CD4 carrier in circulation that is capable of both clearing free HIV and gp120 and binding to HIV-infected cells. The latter could form aggregates that would be removed from circulation by phagocytosis by the RES.

The human red blood cell (RBC) ($t_{\frac{1}{2}}$ ~60 days) appeared as a good candidate for a long-lived CD4 carrier in circulation. We have developed a method of electroinsertion of xenoproteins in the RBC membranes (*Biochim. Biophys. Res. Comm.* 159, 34, 1989; *Biochim. Biophys. Acta* 1027, 53, 1990) without loss of antibody binding properties and stability in circulation. To evaluate the applicability of this approach, we expressed the full length CD4 molecule (which includes the membrane spanning sequence) in insect cells (*spodoptera frugiperda 9*) using the baculovirus expression vector system (*Proc. Natl. Acad. Sci. USA* 86, 7731, 1989). Recombinant CD4 (rCD4) was inserted in rabbit and mouse RBC membranes by electroinsertion. Quantitative assays by flow cytometry of the number of rCD4 epitopes present on the rabbit RBC indicate the presence of 2,000-5,000 epitopes per cell. In a double labelling experiment (RBC - labelled with $Cr^{51}$ and CD4 with $I^{125}$), we found a half life time of CD4 in circulation in rabbits of ~7 days, in mice of 12 days and in pigs of 15 days, in the latter two species $t_{1/2}$ of CD4 equals $t_{1/2}$ of normal RBC.

We also developed low pH-methods of CD4 association with RBC, but the life span in circulation of these RBC-CD4 was curtailed (*Biochim. Biophys. Acta* 981, 51 and 61, 1989). During 30 days of observation, no immune response against either inserted CD4 or glycophorin could be detected in the injected rabbits, whereas the free protein injected i.v. in the same amounts elicited a strong immune response.

To determine whether CD4 molecules inserted into the RBC membrane are functionally equivalent (with regard to their interaction with HIV-1) to native CD4 expressed on the T cell surface, we evaluated three functions normally associated with membrane-bound viral receptors: i) their ability to mediate HIV-1 entry; ii) their ability to inhibit infection of $CD4^+$-T cells with cell-free HIV-1; iii) their ability to mediate the aggregation of RBC-CD4 and HIV-1 infected T cells.

The potential of CD4 exposed on the RBC surface to mediate HIV-1 binding and "entry" was determined using the membrane fluorescence dequenching (DQ) technique. HIV-1 enters CD4-positive T cells and monocytes by fusion between viral envelopes and target cell membranes, and this process can be analyzed quantitatively by measuring an increase in fluorescence resulting from the intermixing between quenched-labelled virions and unlabelled target membranes. As measured by the DQ method, HIV-1 fused in a cell number-dependent manner with human RBC-CD4 but not with control human RBC. Fusion of HIV-1 with RBC-CD4, like that with $CD4^+$ cells, occurred at 37° C but not 4° C and had rapid kinetics with half-DQ time of 5-8 min. at 37° C. It is noteworthy that pretreatment of RBC-CD4 with OKT4A antibodies significantly reduced the DQ. This suggests that the binding and fusion of HIV-1 with RBC-CD4 is mediated by the same functional region(s) of the CD4 molecule that serve as HIV receptor on T cells (*Proc. Natl. Acad. Sci. USA*, in press, 1991).

We tested next whether RBC-CD4 have the capacity to prevent infection *in vitro* with cell-free HIV-1. This test was crucial for the strategy to use RBC-CD4 as competitors with circulating T4 cells in order to reduce the *in vivo* titers of HIV in circulation and to possibly eliminate gp120-expressing, HIV-infected cells from circulation. RBC-CD4, RBC-CD4/OKT4A, RBC, or CEM cells were preincubated with

HIV-1/$III_B$ for 30 min. at 37° C; the cells were removed. Filtered supernatants were then used for infection of target CD4-positive T cells. Pretreatment with either RBC-CD4 or CEM (human T4-lymphoblasts) cells drastically reduced the infectious titer of HIV-1 present in the virus preparation. RBC-CD4 in which HIV-1 binding CD4 epitopes were blocked with OKT4A antibodies were less effective in "clearing" infectious HIV. Similar results were obtained when C-8166 cells, which are very susceptible to HIV infection, served as targets in these experiments.

To evaluate whether RBC-CD4 can interact with HIV-1 infected T lymphocytes, RBC-CD4 were mixed with the chronically infected CEM/N1T-E cells at the numerical ratio of 100:1, respectively, and examined under a light microscope after 5-10 min. of incubation at 37° C. Numerous aggregates between CEM/N1T-E cells and RBC-CD4, but not control RBC, were seen within a short time of incubation and fusion was observed between RBC-CD4 and T cells and occasionally among T cells themselves. Thus, RBC-CD4 appears to recognize the native gp120 molecules on the surface of HIV-1 infected cells, and thus aggregate with these cells (*Proc. Natl. Acad. Sci. USA*, in press, 1991; *J. AIDS* 3, 1041-1045, 1990).

LIPOSOMES AS CARRIERS OF ANTIGENS AND ADJUVANTS. Carl R. Alving, Membrane Biochemistry Branch, Walter Reed Army Institute of Research, Washington, DC 20307-5100 USA.

Liposomes containing lipid A served as carriers for immunizing mice, rabbits, monkeys, and humans against synthetic conjugated or unconjugated peptides, recombinant proteins, and lipid A. Liposomes containing unconjugated peptides induced murine monoclonal antibodies having conformational specificities. Liposomal native lipid A and liposomal monophosphoryl lipid A (MPL) had potent dose-dependent adjuvant properties. An experimental vaccine was developed consisting of liposomes containing both MPL and a recombinant antigen ($R32NS1_{81}$) having epitopes from the repeat region of the circumsporozoite protein of *Plasmodium falciparum*. The liposomes were adsorbed with alum. A phase I safety trial of the vaccine in humans revealed no significant acute toxic effects and the vaccine was nonpyrogenic in humans even at an MPL dose of approximately 2.2 mg. High titers of IgG antibodies both to repeat region epitopes of the *P. falciparum* antigen and to the MPL itself developed within two weeks after a single injection of the vaccine in humans. The highest dose of liposomal MPL induced the highest titers of antibodies to lipid A. Liposomes effectively blocked potential toxic effects but retained the adjuvant effects of lipid A. The vaccine consisting of alum-adsorbed liposomes containing high doses of MPL and encapsulated antigen appears to be safe and efficacious for inducing humoral immune responses in humans.

# Liposomes for cell targeting: current status and prospects.

Lee Leserman
Centre d'Immunologie INSERM-CNRS de Marseille-Luminy
Case 906
13288 Marseille CEDEX 9
France

The goal of many investigators using liposomes is the *in vivo* delivery of liposome associated or encapsulated reagents to specific cells, for the purpose of killing them, modifying their function, or providing genes or gene products which they lack. This idea remains a useful goal, but research over the past decade has indicated that the problems which need to be overcome in order to achieve this goal are more complex than originally appreciated. What is the gap between this objective and that which is obtainable? Specific questions which will be discussed include the following:

1) What are the benefits for delivery into cells of associating a ligand specific for some cell-surface determinant with the liposomes?
2) Is attachment of cell-specific ligands useful in an *in vivo* context?
3) What is the mechanism of entry of liposome contents into cells?
4) What kinds of molecules do we want to encapsulate in liposomes for specific targeting?

Treatment of tumors using cytostatic-containing (immuno)liposomes

G. Storm, Dept. of Pharmaceutics, University of Utrecht, The Netherlands

When drug carriers such as liposomes are considered for more effective drug delivery, two basic issues are involved: sustained release of drugs and targeting. Targeting encompasses two different forms. One is *passive targeting* which relates to the natural distribution patterns of carrier systems. A more specific form of drug targeting is *active targeting*, involving the linkage of some type of a ligand (e.g., a monoclonal antibody) to the drug carrier. Targeting is attained by the recognition at the molecular level through the direct and specific interaction between the recognition site of the drug carrier and the receptor on the target cell. *Sustained release* refers to the potential of liposomes to provide a 'depot' for release of an encapsulated drug over an extended period of time.

These issues, sustained release and targeting, provide the rationales for the use of liposomes for the enhancement of the therapeutic index of antitumor agents. Recent studies show that liposomes can reduce certain types of drug-related toxicity (e.g. doxorubicin cardiotoxicity) with preservation of antitumor activity as a result of modified pharmacokinetics and tissue distribution of the antitumor agent. After intravenous injection of liposomes, the drug can be released from liposomes still being present in the blood directly but also indirectly from the mononuclear phagocyte system (MPS) following uptake and processing by macrophages. Liposomes can be constructed to release the encapsulated drug quickly or in a slow release manner after in vivo administration.

Perhaps the most significant limitation associated with intravenous liposomal drug delivery is the rapid uptake of liposomes by the MPS. Although successful attempts have been made recently in developing liposomes with a strongly reduced affinity for the MPS, their potential for improved site specific delivery still has to be assessed. Another major limitation is that liposomes cannot permeate epithelial and/or endothelial boundaries with the possible exception of endothelium lining certain diseased sites. In the present contribution, we will present work in which these limitations are circumvented by defining as targets intraperitoneally localized tumors, in particular the ascites form of ovarian carcinoma, which should be accessible to liposomes injected directly into the peritoneal cavity. Encapsulation of antitumor drugs in liposomes may alleviate problems associated with local instillation of these drugs. As particles, liposomes are likely to be retained in the peritoneal cavity for longer periods than would most free drugs. Retention of liposomes may be enhanced further by the obstruction of lymphatic drainage that occurs in ovarian carcinoma. Retention of drug-loaded liposomes in the peritoneal cavity would therefore permit sustained exposure of tumor cells to high therapeutically active concentrations of cytotxic agents with, presumably, lower systemic toxicity. In addition, the local inflammation produced by irritant drugs, such as doxorubicin, is reduced by encapsulation in liposomes. Finally, ligands linked covalently to the liposome surface (so-called immunoliposomes) may confer greater tumor-specific cytotoxicity within the peritoneal cavity.

David W. Grainger
Assistant Professor
Department of Chemical and Biological Sciences
Oregon Graduate Institute of Science and Technology
(formerly Oregon Graduate Center)
Beaverton, Oregon 97006-1999 USA

Title: Binding, Interaction, and Organization of Proteins with Lipid Model Membranes

Abstract:

Many amphiphiles self assemble in water to present an array of microstructures that are physically, not chemically, stabilized. This stability is affected by the presence of proteins that associate with the amphiphilic assemblies to produce organized superstructures critical to function in biological systems. Interactions between water-soluble proteins and membrane lipids may be investigated optically using both lipid monolayers at the air-water interface and supported lipid membranes on solid supports. Surface recognition and adsorption processes between solubilized proteins and lipid interfaces can be visualized directly using video-enhanced microscopy of lipid monolayers at the air-water interface as well as total internal reflection fluorescence together with a charge coupled device using supported lipid monolayers.

Using fluorescent haptens immobilized to lipid monolayers, the specific binding reaction of monoclonal antibodies with these haptens results in fluorescent quenching. This recognition can then be spectrometrically followed and compared to data for various physically distinct biomembrane models: monolayers, micelles, and vesicles. Recognition and quenching of the fluorescent hapten is dependent upon lipid chemistry. Binding results in formation of 2-dimensional antibody domains at the air-water interface. Computer simulations modeling protein-membrane interactions are used to understand recognition and quenching reactions.

In a second case, the use of an enzyme substrate as the monolayer-forming lipid together with the enzyme itself as the membrane-active protein combines specific recognition with enzyme function at the biomembrane interface. The hydrolytic action of phospholipase $A_2$ in monolayers is followed visually with fluorescent microscopy. Enzyme hydrolysis of the lipid interface promotes formation of unique enzyme domains in two dimensions in the monolayer after critical extents of monolayer hydrolysis. These protein domains are very stable against monolayer compression and expansion, indicating some structural integrity. Binding of a water-soluble cationic dye to the interface of these hydrolyzed monolayers demonstrates that the enzyme domains are induced by electrostatic interactions with large, negatively charged phase-separated fatty acid domains at the air-water interface.

In a third case, protein adsorption to supported lipid monolayers is followed using total internal reflection fluorescence in combination with a charge-coupled device. Adsorption of albumin and gamma globulin onto supported lipid monolayers of various head group chemistries were followed spatially and kinetically over the surface. Although kinetics are diffusion controlled and independent of the surfaces studied, adsorption levels of both proteins are extremely low compared to control surfaces and suggest appropriate uses of biomimetic surfaces as passivating, compatible biomaterials.

# EQUILIBRIUM, TRANSPORT AND KINETICS OF ANTIBODIES ON PLANAR MODEL MEMBRANES MEASURED BY QUANTITATIVE FLUORESCENCE MICROSCOPY.

N. L. Thompson, M. L. Pisarchick, C. L. Poglitsch, M. M. Timbs, M. T. Sumner, and H. V. Hsieh, Department of Chemistry, University of North Carolina, Chapel Hill, NC, USA 27599-3290

Several aspects of the dynamic behavior of antibodies specifically bound to substrate-supported planar membranes have been characterized with quantitative fluorescence microscopy. First, very slow (~ sec) rotational mobilities of a fluorescently labelled anti-dinitrophenyl monoclonal antibody (ANO2) specifically bound to solid-like Langmuir-Blodgett (LB) monolayers composed of a mixture of dinitrophenyldioleoylphosphatidylethanolamine and distearoylphosphatidylcholine have been characterized by polarized fluorescence photobleaching recovery. The ANO2 rotational mobility was approximately equivalent to the rotational mobility of the fluorescent lipid dioctadecyltetramethylindocarbocyanine incorporated into the monolayers. Second, the equilibrium behavior of fluorescently labelled ANO2 and its Fab at LB monolayers composed of a mixture of dinitrophenylaminocaproyldipalmitoylphosphatidylethanolamine and dipalmitoylphosphatidylcholine has been measured with total internal reflection (TIR) fluorescence microscopy. The results show that intact ANO2 has an apparent association constant only ten-fold higher than that of its Fab, suggesting that a significant fraction of the bound, intact ANO2 is attached by only one antigen binding region at equilibrium. Kinetic studies using TIR illumination with fluorescence photobleaching have demonstrated that the apparent off-rate for ANO2 Fab at the LB monolayers is ~ 0.1-1 $sec^{-1}$ and that the recovery curves are distinctly nonmonoexponential. Third, a mouse IgG Fc receptor (moFc$\gamma$RII) has been purified from a macrophage-related cell line and functionally reconstituted into substrate-supported planar model membranes. TIR fluorescence microscopy has been used to measure and compare the apparent association constants of a variety of fluorescently labelled antibodies with the reconstituted receptors. Supported by NIH grant GM-37145 and NSF-PYI grant DCB-8552986.

LIPIDS MATRICES AND RECEPTOR-LIGAND INTERACTIONS.

G. Graham Shipley. Biophysics Department, Boston University School of Medicine, Boston, MA 02118, U.S.A.

The structure, properties and mutual interactions of membrane phospholipids, glycosphingolipids and cholesterol have been examined using techniques such as X-ray/neutron diffraction, scanning calorimetry, and NMR. Systematic variations in the lipid polar group, interfacial region and hydrocarbon chain structure have led to a reasonable understanding of the molecular factors governing lipid bilayer (crystalline, gel, fluid, interdigitated) and non-bilayer (hexagonal, cubic, micellar) structure. Recent studies of mixed-chain phosphatidylcholines, synthetic cerebrosides and gangliosides will be used to illustrate principles of lipid structure and lipid-lipid interactions. This systematic approach has been crucial for the development of Langmuir-Blodgett lipid transfer techniques to study membrane receptor-ligand systems in oriented arrays. This approach will be illustrated by studies of the ganglioside $GM_1$-cholera toxin system. Oriented 2-dimensional crystals have been formed and electron microscopy-image reconstruction methods are being used to provide structural information on negatively-stained preparations. Finally, we have grown 3-dimensional crystals of the ganglioside-binding subunit of cholera toxin. High resolution crystallographic studies are in progress.

# ROLE OF MEMBRANE COMPONENTS ON ANTHRACYCLINE CYTOTOXICITY.

J. M. Gonzalez-Ros, J. M. Canaves, F. Soto, J. Aleu & J. A. Ferragut.
From the Department of Neurochemistry and Institute of Neurosciences, University of Alicante, 03080 Alicante, Spain.

The plasma membrane of tumour cells is receiving increasing attention in regard to cellular multidrug resistance (MDR). When confronted to cytotoxic agents, MDR cell lines exhibit a reduced intracellular accumulation of the drugs and a decreased sensitivity to these agents, relative to those observed in the parental drug-sensitive cell lines. An increased drug efflux has been proposed to explain this phenomenon, based on the overexpression of certain membrane glycoproteins (the P-glycoprotein family), responsible for actively pumping the drugs out of the cells. On the other hand, several membrane-active agents, including well known calcium channel blockers such as verapamil (VRP), have been shown to increase the sensitivity of MDR cells to the cytotoxic drugs and it has been suggested that the drug-enhancing activity of VRP occurs by competition with the antitumor drugs for binding to common sites on P-glycoproteins. Nonetheless, there are MDR cell lines and tumors which do not express P-glycoproteins and therefore, it should be expected that mechanisms other than those mediated by P-glycoproteins, are also involved in conferring drug resistance to tumor cells and in producing the cellular response to VRP.

In this communication we have studied plasma membranes from wild murine leukemia cells and from stable MDR sublines with primary resistance to daunomycin (DNM), which do not express P-glycoproteins. Freeze-fracture studies of these membranes reveal marked alterations in the architecture of the plasma membrane of the drug-resistant cell lines, thus, confirming that in spite of the lack of P-glycoproteins, modifications of the plasma membrane may also be related to the mechanisms by which these tumor cells become resistant to drugs. We have also used drug binding and fluorescence techniques in isolated plasma membranes and concluded that there is a role for certain lipids, whose presence in the membrane seems to be dependent upon the cellular phenotype, in determining i) the extent of DNM binding to the membrane and ii) the location of DNM within the membrane bilayer. Furthermore, differential scanning calorimetric studies on the interaction between DNM and/or VRP with model lipid vesicles, indicate that VRP prevents, in a concentration-dependent manner, the alterations in the phospholipid phase transition expected from the presence of DNM in the bilayer.

Our results suggest that the lipid bilayer of the plasma membrane could provide appropiate sites where both, the interaction of antineoplastic drugs and the effects of resistance-reverting agents on their activity, can be partly modulated.

# POLYMERIC DRUG-CARRIERS : HAEMATOCOMPATIBILITY

R. Duncan
CRC Polymer-Controlled Drug Delivery Group, Dept. Biological Sciences, University of Keele, Keele, Staffordshire ST5 5BG, England

Soluble polymers designed as targetable drug-carriers for systemic use are usually administered intravenously, and therefore the first biological environment encountered is the blood with its contained proteins (albumin, fibrinogen), enzymes, immunoglobulins, and cells (red blood cells (RBCs), platelets and leucocytes;including granulocytes, lymphocytes and monocytes). In addition the circulating polymer conjugate is exposed to the endothelial cell surface lining the blood vessels, and at sites of discontinuous endothelium (eg.liver and in some cases tumour microvasculature) the conjugate may be able to extravasate and reach other cell types. The latter is essential if such conjugates are to achieve cell-specific delivery of a drug payload.

Biocompatibility has been defined as "the ability of a material to perform, with an appropriate host response in a specific application", obviously haematocompatibility will greatly affect the successful performance of a targeted polymeric drug-carrier. Although a number of pharmacopeal and legislative tests have been documented to define haematocompatibility of implants, particularly vascular prostheses, there is as yet no equivalent for evaluation of soluble polymers being developed for biomedical use.

Obviously, a large portfolio of tests is needed to document fully haematocompatibility in respect of all the complex interactions that can occur. We have recently begun to develop some simple, but quantitative assays that can be used as a preliminary screen. Polymer interactions with RBCs and platelets in vitro have been documented using several techniques: haemoglobin release, changes in light scattering and morphologically (SEM). In addition, polymer toxicity towards a human lymphoblastoid cell line has been monitored using the MTT assay.

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LIPOSOME ENCAPSULATED HEMOGLOBIN; IN-VIVO EFFICACY OF A SYNTHETIC RED CELL SUBSTITUTE.

Alan S. Rudolph[1], Beth Goins[1], Frances Ligler[1], Richard O. Cliff[1], William Phillips[2], Robert Klipper[2], Reuven Rabinovici[3], and Giora Feurstein[3]. [1]Center for Biomolecular Science and Engineering, Naval Research Laboratory, Washington, DC. [2]Department of Nuclear Medicine, University of Texas at San Antonio, San Antonio, TX. and [3]Department of Cardiovascular Pharmacology, Smith-Kline Beecham, King of Prussia, PA.

We are currently involved in a multilaboratory effort to study the safety and efficacy of Liposome encapsulated hemoglobin (LEH) as an artificial oxygen carrying fluid. The circulation persistence of LEH in small animal models at doses which might mimic the clinical use of LEH (an infusion of 10-30% of the blood volume) reveals a two rate removal from circulation over the course of 24 hours. The slower removal period (observed after 4 hours) could indicate that the reticuloendothelial system (RES) is saturated at this given dose. Further evidence of the interaction of LEH with RES is found in the study of the biodistribution of radiolabeled LEH in the rabbit. We have used a lipophilic chelator, hexamethylpropylamino-oxime, to radiolabel LEH with technicium. The radiolabeled LEH is infused into the ear vein of an anesthetized rabbit and the rabbit is imaged under a gamma camera for 2 hours continuously. The rabbit is then housed and imaged again at 20 hours, at which time the animal is sacrificed and the organs weighed and counted for validation of the image distribution. These results indicate that the liver and spleen are the major sites of organ removal, with rapid uptake by the liver two hours post-infusion, while the spleen accumulates LEH more slowly. At 20 hours post-infusion, the liver and spleen show almost equivalent levels of LEH uptake. Most significantly, no significant uptake of LEH occurred in the kidney, a common site of hemoglobin toxicity. This could indicate that no significant release of hemoglobin occurs as LEH is metabolized. Blood chemistry analysis of rodents and rabbits given variable doses of LEH also show no sign of nephrotoxicity as evidenced by normal blood creatinine and BUN levels. Moderate and transient rise in the liver enzymes SGOT and SGPT are observed. Pathological studies of small animals given variable doses of LEH and examined up to 4 weeks post-infusion also show no significant organ pathology. The hemodynamic consequences of giving normovolemic infusions of LEH show a transient thrombocytopenia which is transient and absent at 30 minutes post-infusion. Finally, we have employed water replacement molecules in the freeze-dried preservation of LEH and our animal studies with reconstituted LEH show similar properties to fresh LEH. Continuing effort are underway to evaluate the oxygen carrying efficiency of this material is appropriate hemorrhagic shock models.

# POLIHYDROXIALKANOATES, A FAMILY OF BIODEGRADABLE PLASTICS FROM BACTERIA

Francisco Rodriguez-Valera

Departamento de Genética Molecular y Microbiología,
Universidad de Alicante, Campus de San Juan,
Apdo. 374, 03080 Alicante

The accumulation by certain bacteria of poly-ß-hydroxybutyrate (PHB) as a carbon reserve material has been known for many years. This polymer is a polyester with the properties of a thermoplastic similar to polypropylene. In fact, taking advantage of the inspecificity of the polymerase enzyme, that will incorporate any ß-hydroxy acid into the growing chain, just by providing the adequate precursors in the culture medium, a whole family of polyesters with different mechanical and chemical properties can be synthesized: the so-called poly-ß-hydroxyalkanoates (PHA). These plastics are intrinsically biodegradable and are being introduced into the newly born and rapidly expanding market of biodegradable plastics. The aim is to reduce the dramatic damage inflicted on the environment by petro-chemical plastics due to their extended lifetime in terrestrial and aquatic environments. Several clinical and pharmacological applications are also possible and are being explored.

One essential restraint to the use of bacterial plastics is their production cost. At present, a polymer produced by the eubacterium *Alcaligenes eutrophus* is being exploited by the British conglomerate ICI and commercialized under the trade name of Biopol. The current market price of this product reaches over $20 per kilogram while the equivalent petrochemical is below $1. To reduce production costs we are developing a process which uses an extremophile, the archaebacterium *Haloferax mediterranei* as producer. The peculiar conditions in which this organism grows (more than 20% NaCl is present in the culture medium) preclude contamination by other organisms and allow the development of extremely large and simple production facilities. The organism also shows excellent production parameters and product quality.

# POSITIVE STAINING OF OUTER MEMBRANE LIPOPOLYSACCHARIDES OF GRAM-NEGATIVE BACTERIA: IMAGE ANALYSIS FOR MEMBRANE BIODIAGNOSTICS

J. Hanker*, J. von der Heiden**, H. Patel*, H.D. Peterson*, R.L. Hopfer* and B. Giammara***
*Univ. of North Carolina, Chapel Hill, NC 27599-7455 USA
**Olympus Corp., Precision Instrument Div., Lake Success, NY 11042 USA
***Univ. of Louisville, Louisville, KY 40292 USA

Since the introduction of antimicrobial agents in the early 1940's, septicemia and shock resulting from gram-negative bacteremia have been responsible for an increasing number of deaths from a wide variety of infections.[1,2] The diagnostic problem resulting from poor staining of gram-negative bacteria by the Gram stain is complicated by the difficulty in culturing many of these pathognomonic, especially anaerobic, strains. The PATS reaction, a staining method originally developed in our laboratory for demonstrating basement membrane collagens[3,4], has also been found useful for positively staining gram-negative bacteria.[5,6] This method demonstrates gram-negative but not gram-positive microorganisms because all of the former (but none of the latter) have bacterial lipopolysaccharides(LPS) or endotoxins in their outer cell membranes. The PATS reaction demonstrates gram-negative bacteria because it deposits silver on the LPS or endotoxin present in their outer cell membranes. Gram-negative bacteria are much easier to detect by the black or brown silver stain of the PATS reaction than by the Gram stain which shows them only by the pink Safranin counterstain. It is not unusual for gram-negative bacteria to be noted on a slide or coverslip stained by the PATS reaction when their presence had previously been overlooked on the Gram-stained film. Even the presence of gram-negative bacteria in burn wound frozen sections or touch preparations has been called to our attention by the PATS reaction.

Many times gram-negative bacteria such as Fusobacteria, Bacteroides, cocci or spirochetes can be identified at least generically by light microscopy after PATS staining. If required, an area on PATS-stained light microscope slides or coverslips under study may be mapped and then promptly examined at much higher magnification by the backscattered electron imaging mode of scanning electron microscopy to elucidate a questionable structure.

Discrimination of infection from mere colonization of a tissue site by bacteria can be elucidated by computer-assisted image analysis[7-9] of slides or coverslips stained by the PATS reaction. We have been able to program an Olympus CUE-2 Image Analysis System in our laboratory to discriminate and estimate concentrations or counts of certain types of gram-negative bacteria from control or diseased sites and aid clinicians in diagnosis or appropriate antimicrobial or antibiotic selection. With Gram stained specimens this could be done for gram-positive bacteria but would be difficult to achieve for gram-negative bacteria.

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# HYDROPHOBIC PULMONARY SURFACTANT PROTEINS IN MODEL LIPID SYSTEMS

K.M.W. Keough, J. Pérez-Gil, G. Simatos, J. Tucker, K. Nag, C. Boland, J. Stewart, L. Taylor, S. Taneva, L.A. Allwood, and M. Morrow

Departments of Biochemistry and Physics
Memorial University of Newfoundland
St. John's, Newfoundland, Canada A1B 3X9

Pulmonary surfactant contains two small hydrophobic proteins, SP-B and SP-C, which are important for its physiological actions. SP-C enhances the adsorption of either dimyristoyl- or dipalmitoylphoshatidylcholine (DMPC or DPPC) to the air-water interface, as well as mixtures containing DPPC and either phosphatidylglycerol (PG) or phosphatidylinositol (PI). It is somewhat more effective with DPPC/PI mixtures. SP-C disturbs the packing of the acyl chains, but not the head groups, of disaturated PC. Studies with a model peptide suggest the SP-C may bend calcium. SP-C alters the packing of DPPC in monolayers. The phase of the PC with which SP-C or SP-B is interacted does not influence its structure. SP-B does not substantially alter the order and motion of the acyl chains of DPPC. (Supported by the Medical Research Council of Canada and Ross Laboratories.)

# AXON, SCHWANN CELL, AND COLLAGEN FIBRIL INTERACTIONS IN AVULSIVE PERIPHERAL NERVE INJURY REPAIR WITH A BIODEGRADABLE NEUROPROSTHESIS AND A BASEMENT MEMBRANE GEL

Beverly L. Giammara*,**, John R. Zuniga** and Jacob S. Hanker**
*Analytical Electron Microscopy Laboratory, Univ. of Louisville, Louisville, KY 40292 USA
**Dental Research Center and Biomedical Engineering Dept., Univ. of North Carolina, Chapel Hill, NC 27599-7455 USA

Regeneration in the peripheral nervous system is generally much more successful than in the CNS. In many avulsive peripheral nerve injuries a nerve segment is destroyed or must be removed and the resulting gap must be bridged to effect reanastomosis of the proximal and distal nerve stumps. Studies in our laboratories over the past 3 years have shown[1,2] that an 11 mm gap created in rat sciatic nerve can be effectively repaired by a biodegradable polyester mesh (polyglactin 910, Vicryl™) sleeve which has been filled with a basement membrane gel (Matrigel™). This gel contains a large amount of laminin, and smaller amounts of the other glycoproteins -- collagen IV, various heparan sulfate proteoglycans and entactin/nidogen found in basement membrane (the first extracellular matrix to appear during embryogenesis[3]).

In our studies it was noted that leg sensation and movement were much improved after 30 to 45 days and it was anticipated that the nerves had reconnected. Upon sacrifice this was noted in each animal. Epoxy sections of the repaired nerve were compared with those of the excised segment by use of a basement membrane silver stain developed in our laboratories for light and electron microscopy.[4] This technique stains basement membrane or type IV collagen brown but type III collagen, reticulin and Schwann cells black. Adjacent sections were also compared with other stains for myelin or for axons. Our new silver stain showed that at 30 to 45 days the endoneurial tubes and perineurium had reformed in the repaired nerve. Stains for axons and myelin, however, showed that they were absent in the sections of repaired nerve although present in sections of the excised segment. These observations suggest that type III collagen or reticulin (endoneurial and perineurial) tubes guide axon formation in peripheral nerve repair with this neuroprosthesis and basement membrane gel.

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# ABSTRACTS POSTERS

# INTERACTION OF RETINOL AND RETINOIC ACID WITH PHOSPHOLIPID MEMBRANES AS STUDIED BY DIFFERENTIAL SCANNING CALORIMETRY

A. Ortiz, F.J. Aranda and J.C. Gómez-Fernández

Departamento de Bioquímica y Biología Molecular
Facultad de Veterinaria, Universidad de Murcia
E-30071 Murcia, Spain

Retinoids are essential compounds for the maintainance of health. Their amphiphilic nature suggests that they will locate within the phospholipid bilayer of membranes, which may constitute a site of action.

We have studied the influence of retinol and retinoic acid, two retinoids of major interest, on the main gel to liquid-crystalline phase transition of various phospholipid membranes by means of differential scanning calorimetry.

Both compounds exert perturbing effects on the phase transition of membranes composed of DPPC, DPPE or DPPG. At concentrations up to 40 mol% of retinoid in the membrane, the ΔH enthalpy change is not much affected with respect to the pure phospholipid, indicating a rather superficial interaction. As the concentration of retinol increases the Tc transition temperature decreases. At concentrations of 33 mol% and higher this compound is able to induce phase separations in DPPC membranes, but not in DPPE or DPPG.

The effect of retinoic acid is much weaker, the Tc and ΔH remaining almost unaltered and equal to that of the pure phospholipid up to concentrations of 30 mol% at neutral pH. Retinoic acid exerts a pH-dependent effect. As the pH decreases, and therefore increases the extent of protonation of retinoic acid, the perturbation of the membrane induced by this compound is less. A strong effect, both on Tc and ΔH, is observed at pH 10, where the retinoic acid moiety will be mainly unprotonated and the negative charge will generate repulsive forces, thus destabilizing the membrane.

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Abstract Poster No.2

# PURIFICATION AND SOME PROPERTIES OF *E. coli* α-HAEMOLYSIN

Helena Ostolaza, Borja Bartolomé*, Iñaki Ortiz de Zárate, Fernando de la Cruz* and Félix M. Goñi.

Dpt. of Biochemistry, University of the Basque Country, Bilbao, Spain, and

*Dpt. of Molecular Biology, University of Santander Medical School, Santander, Spain.

*E. coli* α-haemolysin appears to be related to the pathogenic properties of some strains of this microorganism. This toxin is also interesting from the point of view of biotechnology, since it is one of the few proteins extracellularly excreted by *E. coli*, therefore it can be used in the cell export of other gene products in the form of chimeric proteins. α-Haemolysin purification has found a number of difficulties in the past; in this communication, we present an improved, straightforward purification procedure. The protein, containing a single 110 kDa polypeptide, exists in the form of large aggregates, held together mainly by hydrophobic forces. In the presence of urea or other chaotropic agents, the size of the aggregates decreases, while the specific activity is increased. α-Haemolysin releases the aqueous contents of phospholipid vesicles; the mechanism of bilayer permeabilisation appears to involve non-lamellar lipid intermediates.

# LIPID ENVIRONMENT MODULATION OF CONFORMATIONAL TRANSITIONS OF NATIVE AND MODIFIED GRAMICIDIN A AND STRUCTURAL ANALOGS

Lorenzo Braco, David Salom, Mari Carmen Bañó and Concepción Abad.
*Dept. Bioquímica y Biol. Molecular, Universidad de Valencia, Burjassot, E-46100 Valencia, Spain.*

The conformation of the transmembrane channel forming hydrophobic pentadecapeptide gramicidin A (GA), in organic solvent and especially in phospholipid model membranes, has been the subject of extensive study in a number of laboratories for the last two decades. It has been thoroughly demonstrated and seems at present well established that the head-to-head dimer is actually the predominant configuration of the active transmembrane cation channel (1). However, it has been recently proved that this conformation may not be immediately adopted upon incorporation of the peptide into the lipid environment (2). In fact, we have recently proposed an HPLC strategy which demonstrates that the actual configuration of the peptide in the freshly prepared model membranes is strongly dependent on the "history" of sample preparation so that variables such as nature of the organic solvent, lipid/peptide mole ratio, sonication time or heat treatment can become crucial in determining the inserted GA conformation (3,4). Moreover, we have elucidated the nature of the slow, temperature-dependent conformational transition of the peptide reported to occur in the lipid environment upon incubation of the model membranes (4).

In the present communication we have exploited the aforementioned chromatographic methodology (in combination with other spectroscopic methods) to extend the study in several directions. First, we have analyzed how the peptide conformational transitions and final configuration in the SUV can be affected by membrane composition, e.g., type of phospholipid polar head, hydrocarbon chain length and unsaturation degree, presence of cholesterol and fluidity perturbing agents such as anesthetics, etc. Second, since GA has been shown to be able of inducing hexagonal ($H_{II}$) phases and other types of non-bilayer structures from lipid bilayers (5)(these lipidic "particles" being regarded as reversed phospholipid micelles sandwiched between monolayers of the lipid bilayer), we have studied the conformational behavior of GA in Aerosol OT (AOT)/isooctane reversed micelles as a simple but interesting model for these non-lamellar phases. Third, we have investigated to which extent the peptide conformational behavior can be altered by chemical modification (e.g., N-formylation) or replacement of one or more of the tryptophans by other hydrophobic amino acids (e.g., phe, tyr). The results are interpreted in relation to the peptide function as an ionophore.

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# INTRACELLULAR FUSION OF INFLUENZA VIROSOMES AS ASSESSED BY CYTOPLASMATIC TOXIN DELIVERY AND LIPID MIXING

**R. Bron, A. Ortiz* and J. Wilschut**
Lab. of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands;
*Present address: Dept. of Biochemistry and Mol. Biology, Veterinary Faculty, University of Murcia, Murcia, Spain.

Infectious entry of influenza virus into cells involves uptake of intact virions by receptor-mediated endocytosis, followed by fusion of the viral envelope with the membrane of the endosomal cell compartment. This fusion process, which is mediated by one of the viral spike glycoproteins, the hemagglutinin (HA), is induced by the mildly acidic pH in lumen of the endosomes. We have followed the kinetics of this intracellular fusion process in BHK-21 cells using reconstituted viral envelopes (virosomes; see Stegmann et al., EMBO J. 6, 1987, 2651-2659) as a model for the native virus. Two strains of influenza A virus were used for virosome preparation: X-31, which has a low pH-threshold for fusion (pH 5.4), and NIB-24, with a high pH-threshold of fusion (pH 5.9). The following assays were used to follow the intracellular fusion process. First, cytoplasmic delivery of virosome-encapsulated toxins (Diphtheria toxin A chain (DTA) and gelonin), as assessed by determination of the inhibition of cellular protein synthesis. Second, mixing of virosomal lipids with cellular target membrane lipids, as reflected by a decrease of pyrene excimer fluorescence upon dilution of pyrene-labeled phosphatidylcholine (pyrene-PC), coreconstituted in the virosomal membrane. Both toxin delivery and pyrene-PC dilution were inhibited by 20 mM $NH_4Cl$ or by pretreatment of the virosomes alone at low pH. NIB-24 virosomes fused with a relatively short lag time (5-10 min) after internalization from the plasma membrane, whereas X-31 virosomes fused after a lag time of at least 15 min. The fusion of NIB-24 virosomes, in contrast to that of X-31 virosomes, was not abolished by treatment of the cells with 10 μg/ml nocodazole, an inhibitor of transport from early to late endosomes. These data suggest that influenza virus strains fuse at different sites of the endocytic pathway (early or late endosomes), depending on the pH-dependence of fusion.

Our results demonstrate that virosomes can serve as useful models to study viral entry into cells. Moreover, this study reveals attractive possibilities for application of influenza virosomes as a carrier system to introduce foreign water-soluble or membrane-associated bioactive molecules into cells.

# EXCITON INTERACTION OF POLYENE ANTIBIOTICS PROMOTED BY CHOLESTEROL IN SOLUTION AND IN A MODEL SYSTEM OF MEMBRANE.

M. Castanho, A. Coutinho, M. Prieto
Centro de Química-Física Molecular
Complexo I, I.S.T.
1096 Lisboa Codex, Portugal

The alterations in the absorption (namely the ratio between 0 ← 0 and 2 ← 0 transitions intensities) and excitation fluorescence spectra observed for the polyene antibiotics filipin and nystatin in the presence of cholesterol are due to an exciton interaction between the polyenes. In this way these alterations cannot be used to evaluate the antibiotic-sterol "affinity". Filipin and nystatin molecules in aqueous solutions partitionate into the sterol micelles, these structures being very efficient to induce exciton interaction (stacked arrangement of chromophores). For filipin incorporated in lipid bilayers (SUV of DPPC), the sterol is able to induce the same type of aggregate, at variance with nystatin. The strict requirements for exciton interaction (chromophore distances and orientations) provide direct information on the structure of this antibiotics when incorporated in membranes containing sterol.

# THE ACCUMULATION OF BASIC PEPTIDES WITHIN LARGE UNILAMELLAR VESICLES EXHBITING A PROTON GRADIENT: A SURVEY

Ajoy C. Chakrabarti[1], Ian Clark-Lewis[1,2] and Pieter R. Cullis[1,3]

[1] Department of Biochemistry, [2] Biomedical Research Center, University of British Columbia, Vancouver, B.C., Canada, V6T 1W5.
[3] The Canadian Liposome Co. Ltd., North Vancouver, B.C., Canada, V7M 1A5.

The presence of a transmembrane pH gradient (7.5/4.0; exterior/interior) results in the rapid and efficient accumulation of various basic peptides (both naturally occuring and model peptides) into large (100 nm) unilamellar egg phosphatidylcholine vesicles (LUVs). Two peptides that were studied in detail were lysine methyl ester and a hydrophobic pentapeptide (Ala-Met-Leu-Trp-Ala) made as a methyl ester or amide. Activation energies for the transbilayer movement of some of these peptides were calculated to be approximately 30 kcal/mole. Experiments with several of these peptides have established that they are transported in a manner consistent with the permeation of the neutral (deprotonated) form. It is suggested that this property may have general implications for the mechanism of transbilayer translocation of peptides, such as signal sequences, which exhibit weak base characteristics.

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# THERMAL DENATURATION OF MYELIN MEMBRANE PROTEINS

J. Ruiz-Sanz[1], J. Ruiz-Cabello[2], P.L. Mateo[1] and M. Cortijo[2].

[1] *Departamento de Química Física, Facultad de Ciencias, Universidad de Granada, E-18071-Granada, Spain.*

[2] *Departamento de Química Física II, Facultad de Farmacia, Universidad Complutense de Madrid, E-28039-Madrid, Spain.*

Myelin membrane plays a crucial role in nervous transmission and is related to human diseases such as multiple sclerosis and severe encephalomyelitis. We have studied the thermal behaviour of this membrane using various different physical techniques.

Differential scanning calorimetry (DSC) experiments with myelin at pH 7.0 show a main transition centered at 80.3±0.2 °C with a Δh of 4.7±0.6 J/g of protein. The transition is irreversible and kinetically controlled, and has been put down to protein denaturation by means of FT-IR experiments. Thermal gel analysis (TGA) measurements indicate that the transition corresponds mainly to the irreversible thermal denaturation of myelin proteolipid (PLP); DM-20 protein, which represents only about 1/5 of the PLP content, also undergoes denaturation at around 80 °C. The Wolfgram Fraction denatures at 55 °C-60 °C, while the TGA technique has been unable to detect myelin basic protein denaturation.

Since PLP is traditionally purified by chloroform-methanol mixtures we have studied the effect of these solvents on the above DSC transition. In fact, at organic solvent concentrations lower than 10% (v/v) the DSC transition disappears after removal of the organic mixture. On the other hand, the DSC transition remains after treating the membrane with detergents, although the enthalpy and Tm values are lower. TGA shows that this new transition can also be assigned to PLP denaturation. Detergents thus seem to be very suitable for PLP purification.

# Influence of PE on $Ca^{2+}$-induced fusion of NAPE-containing liposomes.

F.Rosell, S.Sempere, M.Mora, M.A.De Madariaga.
Department of Biochemistry & Physiology. University of Barcelona.

The fusion studies carried out with liposomes as a model system enable us to make an approach in order to understand the possible role of the different lipids in the biological systems, eliminating thus the complexity of the analysis and at the same time simplifying it.

In previous studies we have revealed $Ca^{2+}$, $Mg^{2+}$ and polylysine ability to induce the fusion of liposomes that contain N-acyl derivatives of PE (NAPEs) when they are present in molar fractions higher than 0.5. Phosphatidylethanolamine (PE) is a neutral lipid with a well-known capacity to accelerate $Ca^{2+}$-induced fusion of vesicles containing acidic phospholipids (PS, PA), and is also the biological precursor of NAPE. Both of these characteristics have led us to study the effect of their presence in the fusion of NAPE-containing liposomes.

The aggregation has been followed by measuring the increase of absorbance at 400 nm. The fusion has been measured by the RET method with NBD-PE and Rho-PE and has been confirmed by the assay of Tb/DPA.

The presence of PE in NAPE liposomes increases the rate of the fusion, probably due to a dehydrating effect and the formation of non-lamellar phases related to the appearance of the fusion intermediaries.

The substitution of PE molecules for their N-acyl derivatives in the PC:PE (1:1) system raises the rate of aggregation which should logically be related to the increase in the superficial density of negative charge. The non-linear response, which presents a sudden increase from 0.25 molar fraction, would be in agreement with the preferential localization of NAPE on the inner side of membrane.

The replacement of PC for PE molecules in PC:NAPE 1:1 liposomes also causes an increase in the aggregation and the fusion, and at same time a decrease in the threshold concentration.These facts can be related, as before, to the dehydrating effect of PE, but also to a removal of the NAPE from the inner side towards the outer one of the membrane.

These results suggest a possible physiological role of NAPEs in highly degenerative processes, such as myocardial infarct, in which its presence could contribute to the disruption of the cellular membranes through a process of fusion and internal vesiculation.

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# PENETRATION OF BETAXOLOL LOADED NANOCAPSULES IN LIPID MONOLAYERS AS ARTIFICIAL CORNEA MEMBRANE MODEL

M.A. Egea*, O. Valls*, M.A. Alsina*, M.J. Alonso**, C. Losa** J.L. Vila-Jato**, y M.L. Garcia*

* Physichochemical Unity. Faculty of Pharmacy. University of Barcelona

** Laboratory of Pharmaceutical Technology. Faculty of Pharmacy University of Santiago de Compostela.

The ß-adrenergic blocking agents are the most commonly prescribed, to reduce intraocular pressure, in topical treatment of glaucoma. Althought these agents are relatively well tolerated, adverse systemic reactions, involving cardiac and pulmonary system, have been reported. Betaxolol hydrochloride, a $ß_1$-cardioselective beta-blocker introduced in therapeutic recently, offers certain advantages for use in patients with advanced cardiovascular and obstructive airway disease, due its relative selectivity for $ß_1$-adrenergic receptors and its favorable pharmacokinetic properties.

In the glaucoma tretment, the drug must traverse the cornea to reach the aueous humor and, generally the percentatge of the applied dose which reach the anterior segment ranges from 1-2%. In order to improving the topic bioavailability of betaxolol, leaing to a reduction of the amount of the applied drug and minimizing systemic adverse effects, different kinds of ocular drug delivery system, sucha as liposomes, nanocapsules or nanoparticles, have been developed in the last years.

This work was undertaken to analyze the penetration kinetics of betaxolol loaded or unloaded biodegradable nanocapsules in lipid monolayers with the same lipid composition as cornea. This lipid composition of monolayers has been chosen because the cornea acts as a first biological barrier that must be crossed by drug to reach anterior segment of the eye. The possible interactions of the stabilizing agents used in the nanoparticle formulation with the lipids above mentioned were also investigated.

A different behaviour was constated when several amounts of betaxolol loaded nanocapsules was injected into the subphase in relation with free drug or free nanocapsules. The interaction of these producsts with lipid artificial cornea membrane was measured in all cases as variations of surface pressure increases.

# PROTEIN STABILITY AND INTERACTION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR WITH CHOLINERGIC LIGANDS STUDIED BY FOURIER-TRANSFORM INFRARED SPECTROSCOPY

G. Fernandez-Ballester[a], J. Castresana[b], J. L. R. Arrondo[b], J. A. Ferragut[a] & J. M. Gonzalez-Ros[a].

From the (a) Department of Neurochemistry and Institute of Neurosciences, University of Alicante, 03080 Alicante and (b) Department of Biochemistry, Faculty of Sciences, University of the Basque Country, 48080 Bilbao, Spain.

The conformational-dependence of the amide I infrared (IR) band has been used to probe the structure features of proteins present in native membranes from *Torpedo* enriched in Acetylcholine Receptor (AcChR). The interference of water IR absorbance on the amide I spectral region has been eliminated through convenient methods of exchange with deuterated water.

The temperature-dependence of the IR spectrum indicates a massive loss of protein secondary structure, occurring at temperatures similar to those reported by differential scanning calorimetry for thermal denaturation of the AcChR and by thermal inactivation of α-Bungarotoxin binding sites on the AcChR (A. Artigues, M. Villar, J. A. Ferragut & J. M. Gonzalez-Ros (1987) Arch. Biochem. Biophys. 258, 33-41), thus, indicating that the observed IR spectral changes, are mostly due to alterations in the structure of the AcChR protein. Furthermore, the presence of detergents as well as that of cholinergic agonists and antagonists, produces also spectral changes which are consistent with the alterations in the protein structure expected from previous calorimetric studies with the AcChR under similar conditions. As different from the information obtained from calorimetric techniques, IR spectroscopy allows to distinguish the contribution of secondary structural changes to the overall change in protein structure. Thus, the prolongued exposure to cholinergic agonists, which stabilize the AcChR protein into the desensitized state, produces relatively small alterations in the β-strand contents of the protein secondary structure, but increases substantially the protein stability, suggesting that rearrangements in the protein tertiary or quaternary structure are more likely to occur than extensive changes in secondary structure, as a consequence of AcChR desensitization.

Use of reconstituted vesicles containing the purified AcChR as the only protein component, allows for studying the effects of specific lipids on the protein structure. We found that cholesterol, a membrane lipid required for optimal AcChR functionality, induces a variety of changes in structural features of the protein. We conclude that the structural changes induced by cholesterol or by other neutral lipids present in soybean lipids extracts, are essential to enable the AcChR to respond properly to cholinergic stimulation.

"IMMOBILIZATION OF HEPATIC MICROSOMAL CYTOCHROME P450 FROM PHENOBARBITAL TREATED RATS IN HOLLOW FIBER BIOREACTORS".

P. Fernandez-Salguero*, A.W. Bunch# and C. Gutierrez-Merino*
* Depto. de Bioquímica y Biología Molecular. Fac. Ciencias. Univ. de Extremadura. 06080-Badajoz. Spain.
# Biological Laboratory. University of Kent at Canterbury. Kent CT2 7NJ. United Kingdom.

The hepatic cytochrome P450 monooxygenase system is the most important membrane complex involved in the detoxification of many lipophilic metabolites, such as steroid hormones and xenobiotics (pesticides and herbicides among them)(Coulson et al. (1984) TIBS 9, 446-449). On the other hand, specific isoenzymes of cytochrome P450 can be selectively induced by chronic treatment of animals (usually rats) with xenobiotics (Thomas et al (1987) Biochemistry 26, 2280-2289; Astrom and DePierre (1986) Biochim. Biophys. Acta 853, 1-27). In addition, hollow fiber bioreactors have been successfully used in the immobilization of cells and enzymes (Bunch A.W. (1988) J. Microb. Methods 8, 103-109; Estrada-Diaz et al (1989) Enzyme Microb. Technol. 11, 725-729).

In this communication, we report the results obtained in the immobilization in Romicon PM10 anisotropic hollow fibers of hepatic microsomes prepared from phenobarbital treated rats, using 7-ethoxycoumarin as substrate. The reactor was operated in transverse mode (from the shell side to the lumen) and mounted on an open circuit in which the activity was measured by taking aliquots at different times intervals. The results show that the maximum activity increases when the flow rate is decreased. In this regard, the activity obtained at 30 ml/h is about 40-50% with respect to that of the soluble enzyme. The titration of the immobilized enzyme with substrate shows a Michaelis pattern, with a Km value quite close to that of the soluble enzyme. This is in contrast to the results obtained by covalently coupling the enzyme to activated polyacrylamide, for in this latter case an increase of the Km with respect to that of the soluble enzyme is observed. The effect of the pesticides warfarin and 3-amino-1,2,4-triazole on the activity of the immobilized system upon the concentration of 7-ethoxy coumarin is in good agreement with the results obtained using the soluble enzyme. The stability of the immobilized system with time is increased with respect to the soluble enzyme, retaining about 90-100% of the initial activity after 24 h of continous operation at 25°C. In conclusion, hollow fiber bioreactors of cytochrome P450 can be a useful model system to test the ability of different organisms to transform xenobiotics, and eventually to help clearance of xenobiotics from the blood of intoxicated mammals.

# STABILIZATION OF LIPOSOMES WITH HYDROGELS

M.J. Fonseca, M.A. Busquets, I. Haro, F. Reig. Laboratório de Péptidos. CID-CSIC. 08034 Barcelona, Spain.

Liposomes are being widely studied as a drug delivery system. In an aqueous liposome dispersion phospholipids can hydrolyse to free fatty acids and lysophospholipids, or oxidise if non-saturated lipids are involved in its composition. Moreover, the entrapped material can be released at a certain extent upon storage. Therefore, the long term chemical and physico-chemical stability of liposome based formulations has become an important issue.

In general the above cited problems can be overcome by the use of saturated phospholipids and high contents of cholesterol (up to 50%). Nevertheless, leakage and agregation are not completely solved.

In the present paper the stability (permeability) of liposomes was studied in the presence of a hidrogel. The study was performed on liposomes made of hydrogenated egg phosphatides and cholesterol (1:1), The Tc of the phosphatides ranged between 45-55°C. The leakage of the entrapped material was determinated by using CF as a fluorescent marker. Three types of liposomes were prepared: multilamellar, small unilamellar and the ones prepared by the dehydration-rehydration method (DRV). Alicuots of these dispersions were mixed with hydrogel solutions of differents concentrations (0, 0.2, 0.4, 0.8% w/w). The final mixtures have increasing viscosities according to the hydrogel content The samples were stored at 5, 15, 23, 40, 50 and 60°C, and CF latency determined as a function of time.

The results obtained show that the size and the number of bilayers seem to have small influence on the leakage process. On the contrary, the presence of a highly viscous medium surrounding the liposomes has a positive effect for samples stored at temperatures under Tc. But, CF leakage was higher for liposomes-hydrogel mixtures when the temperature of the incubation was higher than Tc. This fact could be attributed to the presence of a phase change and/or to the chemical reaction between the lipid components of the liposome and the functional groups of the hydrogel. The presence of lysophosphatides and the cholesterol degradation is now being checked by HPLC analysis.

# APPLICATION OF THE THEORY OF THE VESICULAR GERM FORMATION TO DLPC,DMPC,DPPC AND DSPC PHOSPHOLIPIDS.

C. Llácer, A. O. Vila, M. J. Martos, J.E. Figueruelo and F. J. Molina.
Unitat D'Investigació de Coloides. Dpt. Química Física. Universitat de Valencia.
Avda Blasco Ibáñez, 13, 46010 Valencia. Spain.

The Theory of the Vesicular Germ Formation (TFGV) has been applied to phospholipid systems constituted for Dilauryl-(DLPC), Dimyristoyl-(DMPC), Dipalmitoyl-(DPPC) and Diestearoylphosphatidylcholine(DSPC) respectively. For that, to determinate both the excess of the chemical potential and the surface tension at the formation critical concentration (ccf) has been neccesary The excess of the chemical potential has been obtained experimentally through determination of the formation critical concentration, while the surface tension has been obtained directly. Using the so called "Energetic Balance" (δ) paramater value, for the four phospholipid systems, it is possible to establish the physical and chemical conditions for the formation of liposomes. Under such experimental conditions, according to TFGV , it is possible to predict the spontaneous formation of vesicles all systems estudied, and it is possible also to show the values of the surface tension which transform the characteristics of liposomes notably.

The following table show the most important results corresponding to the unilamellar germs of phospholipid systems estudied.

| System | T (°K) | $a_0 \cdot 10^{10}$ (m) | $V_m \cdot 10^4$ ($m^3$) | σ (N/m) | $\Delta\mu^e$ (Kj/mol) | $ccf \cdot 10^7$ (mol/l) | $\Delta\mu'$ (Kj/mol) | $\delta_{exp}$ | $\rho_{unilam}$ | Q |
|---|---|---|---|---|---|---|---|---|---|---|
| DLPC | 298 | 30,8 | 3,90 | 0,0641 | 81,166 | 10,7 | 37,172 | 4,587 | 1,100 | 252 |
| DMPC | 298 | 35,9 | 4,54 | 0,0683 | 86,574 | 9,9 | 42,188 | 4,884 | 1,024 | 276 |
| DPPC | 318 | 41,0 | 5,19 | 0,0646 | 81,774 | 7,3 | 33,817 | 4,142 | 1,277 | 692 |
| DSPC | 328 | 46,0 | 5,84 | 0,0612 | 77,697 | 5,7 | 26,380 | 3,390 | 3,618 | 12.350 |

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# INTERACTION BETWEEN METHOTREXATE LOADED NANOPARTICLES AND LIPID MONOLAYERS AS MEMBRANE MODEL OF RETICULOENDOTHELIAL SYSTEM

M.I.Llovet, M.L García, M.A.Egea, M.Espina, O.Valls, M.A.Alsina

Department of Pharmacy (Phisicochemical Unity).Faculty of Pharmacy Barcelona University.Barcelona (Spain).

Citotoxic agents, as methotrexate (MTX), are a major therapeutic approach for the treatment of a greater number of localized and metastasized cancers.

Association of methotrexate with polyalkylcyanoacrylates (PACA),by an emulsion-polymerisation procedure, constitute a biodegradable drug delivery system that contribute to improve its penetration into the cellular membranes and could reduce side effects by altering pharmacokinetic disposition of the drug.

The aim of this work was to analyse the penetration kinetics of methotrexate loaded or unloaded polyalkylcyanoacrilate nanoparticles, with the same components as lipids present in the cellular membranes of the reticuloendothelial system spread as a monolayer at liquid-gas interface. These measurements give valuable information about the interfacial behaviour of membrane compounds and also on interactions drug-membrane correlated with phenomena at biological membranes.

Lipid composition of monolayers has been chosen because a major limiting factor to the systemic use of particulate drug delivery systems is the rapid clearance of the carrier from the circulation by the reticuloendothelial system. The possible interactions of the surfactant used in the nanoparticle formulation with the lipids above mentioned were also investigated.

Experiments carried out when the lipid monolayer had not been spread on the surface (0 mN $m^{-1}$ of initial surface presure) show a high pressure increase for MTX/sodium lauryl sulfate loaded nanoparticles againts the free complex. This effect was more important when sample was not centrifugated. This behaviour was similar at different initial surface pressures (0-20 $mNm^{-1}$) of lipid monolayer.

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# DETERMINATION OF AMPHIPHILE NUMBER IN LIPOSOMES ACCORDING TO THE THEORY OF THE VESICULAR GERM FORMATION

M.J.Martos, C.Llácer, A.O.Vila, J.E.Figueruelo and F.J.Molina.
Unitat d'Investigació de Coloides, Dpt Química Física. Universitat de Valencia.
Avda Blasco Ibáñez 13, 46010 Valencia. Spain.

For the determination of amphiphile number in "micelles" all the authors introduce the conditions of chemical equilibrium (1-4), but they are not the aplicable conditions to liposomes according to The theory of the Vesicular Germ Formation (TFGV)(5-6) because the liposomes are one metastable state. Consistently the TFGV stablishes a novel metodologie for the calculation of amphiphile number in the metastable conditions. The following expression shows the formula for the calculation of amphiphile number under TFGV:

$$g = \frac{4\pi e_0^2 \sigma N_A \delta_{ML} \sum_{n=1}^{n} (p_n^2 - p_n + \frac{1}{3})}{\Delta\mu'_{ML}} \qquad (12)$$

where $e_0$ is the thickness bilayer, $\sigma$ is the surface tension of medium , $N_A$ is the Avogadro's number, $p_n$ is the equivalent radius of n-esime cape, $\delta_{ML}$ is the energetic balance of multicape structure: $\delta_{ML} = \Delta'\mu_{ML} / v_m \sigma$, and $\Delta'\mu_{ML}$ is the excess of chemical potential in multilamellar vesicle. The imposition of critical conditions when the energetic barrier value is anulated permit us for the following expression:

$$g^* = \frac{20}{3} \frac{\pi e_0^2 \sigma N_A \sum_{n=1}^{n} (2p_n^2 - 2p_n + 1)}{\Delta\mu'_{ML}} \qquad (15)$$

being $g=g^*$ in the condition critical.

The following expressions shows the amphiphile number value when the watery interlamellar cape is equal at bilayer value ($e_0' = e_0$) and the amphiphile number value when the watery interlamellar cape is null ($e_0'=0$) respectively:

$$g^*_{(e_0' \neq e_0)} = \frac{20}{3} \frac{\pi e_0^2 \sigma N_A}{\Delta\mu'_{ML}} \left\{ (2p_1^2 - 2p_1 + 1)n + (8p_1 - 4) \sum_{n=1}^{n} (n-1) + 8 \sum_{n=1}^{n} (n-1)^2 \right\}$$

$$g^*_{(e_0' \neq 0)} = \frac{20}{3} \frac{\pi e_0^2 \sigma N_A}{\Delta\mu'_{ML}} \left\{ (2p_1^2 - 2p_1 + 1)n + (4p_1 - 2) \sum_{n=1}^{n} (n-1) + 2 \sum_{n=1}^{n} (n-1)^2 \right\}$$

**Acknoledgments.**- Authors are indebted to CICYT .(Project FAR/0768)

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# MULTILAMELLAR VESICLES ACCORDING TO THE THEORY OF THE VESICULAR GERM FORMATION

F.J.Molina, M.J.Martos, C.Llácer, A.O.Vila and J.E.Figueruelo.
Unitat d'Investigació de Coloides, Dpt Química Física. Universitat de València.
Avda Blasco Ibáñez 13, 46010 València. Spain.

The natural tendency of many amphiphilic systems to the formation of multilamellar vesicles shows in results obtained from The theory of the Vesicular Germ Formation(TFGV). So that the penetration degree of system in metastable state give smaller $\rho$ value in multilamellar vesicles instead for unilamellar vesicles. It is possible one estimation of penetration degree in metastable state by considering the aggregates of many capes as groups of single unilamellar vesicles, by the expression: $\rho_n = \rho_1 + (1 + e_0'/e_0)(n-1)$ where $\rho_1$ and $\rho_n$ are the first bilayer equivalent radius and the n-esime bilayer equivalent radius respectively, n is the bilayer number, $e_0$ is the thickness bilayer and $e_0'$ is the watery interlamellar thickness bilayer.

Energetic barrier for multilamellar vesicles is:

$$\epsilon_{ML} = \frac{5}{3}\sum_{n=1}^{n}(2\rho_n^2 - 2\rho_n + 1) - \delta_{ML}\sum_{n=1}^{n}(\rho_n^2 - \rho_n + \frac{1}{3}) \qquad (2)$$

where $\delta_{ML} = \Delta'\mu_{ML}/v_m\,\sigma$ and $\Delta'\mu_{ML}$ is the excess of chemical potential in multilamellar vesicle, $v_m$ is the molar volume of constituent amphiphiles of bilayer and $\sigma$ is the surface tension of medium.

Supposing now two extreme distributions: $e_0'=0$ and $e_0' = e_0$, and by considering the critical conditions of energetic barrier, then it is necessary the energetic barrier is null in multilamellar vesicles, also in unilamellar vesicles. The $\delta_{ML}$ values in these critical conditions are:

$$\delta^*_{ML(e_0'=0)} = \frac{5}{3}\,\frac{(2\rho_1^2 - 2\rho_1 + 1)n + (4\rho_1 - 2)\sum_{n=1}^{n}(n-1) + 2\sum_{n=1}^{n}(n-1)^2}{(\rho_1^2 - \rho_1 + \frac{1}{3})n + (2\rho_1 - 1)\sum_{n=1}^{n}(n-1) + \sum_{n=1}^{n}(n-1)^2}$$

$$\delta^*_{ML(e_0'=e_0)} = \frac{5}{3}\,\frac{(2\rho_1^2 - 2\rho_1 + 1)n + (8\rho_1 - 4)\sum_{n=1}^{n}(n-1) + 8\sum_{n=1}^{n}(n-1)^2}{(\rho_1^2 - \rho_1 + \frac{1}{3})n + (4\rho_1 - 2)\sum_{n=1}^{n}(n-1) + 4\sum_{n=1}^{n}(n-1)^2}$$

Both $\delta^*_{ML}(e_0'=0)$ and $\delta^*_{ML}(e_0'=e_0)$ have tendency towards 10/3 value as when $\rho_1$ has tendency to infinite for one determinated n value, as when n has tendency to infinite for one determinated $\rho_1$ value.

**Acknoledgments.**- Authors are indebted to CICYT.(Project FAR/0768)

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SERUM HIGH-DENSITY LIPOPROTEIN PRE AND POST VENOUS OCCLUSION, G. Monte, E. Melissari, & VV Kakkar, Thrombosis Research Unit King's College School of Medicine & Dentistry, Denmark Hill, London SE5 8RX.

Is now clear that plasma lipoproteins are found in the wall of normal artery and can be transported through endothelium. Both Low-density lipoprotein (LDL) and High-density lipoprotein (HDL) appear to be present in peripheral lymph. It is therefore appropriate to consider the regulation of cholesterol synthesis and metabolism of lipoproteins in arterial endothelium and smooth muscle. In our experiment we measured serum HDL before and after 7 minutes application of venous occlusion by tourniquet in ten healthy male volunteers, aged 20-29 years. From our results we found in post-venous occlusion samples a significant increase in HDL-C and HDL2-C of 20% ($p<0.05$) and 35% ($p<0.05$) respectively, in comparison with pre-venous occlusion samples.

| | HDL-C (mmol/l) | $HDL_2$-C (mmol/l) | Cholesterol (mmol/l) | Triglycer. (mmol/l) | LDL-C (mmol/l) |
|---|---|---|---|---|---|
| PRE V.O | 1.02 | 0.42 | 4.9 | 1.75 | 2.53 |
| POST V.O. | 1.22 | 0.56 | 5.17 | 1.87 | 2.67 |

We concluded that the effect of perturbation of vessel wall by venous occlusion increases the high-density lipoproteins levels.

# IN THE PRESENCE OF PIG LUNG HYDROPHOBIC SURFACTANT PROTEINS, DPPC/PI MIMICS PULMONARY SURFACTANT PHYSICAL PROPERTIES BETTER IN VITRO

J. Pérez-Gil[1], J. Tucker[1], G. Simatos[1] and K.M.W. Keough[1,2]

Dpt. of Biochemistry[1] and Discipline of Pediatrics[2], Memorial University of Newfoundland, St. John's, Newfoundland, CANADA A1B 3X9

Hydrophobic surfactant proteins were isolated from pig lungs and mixed with some simple lipids in order to test their physical properties in vitro. The goal of the present study was to see if the natural properties of the surfactant material could be mimicked by simple mixtures, giving information about the specific lipid-protein interactions responsible for the behaviour of surfactant in vivo.

The mixtures tested were: pure DPPC, DPPC/"egg"PG in 9:1 and 7:3 ratios, and DPPC/yeast PI also in 9:1 and 7:3 ratios. All the samples were assayed in the absence and in the presence of 0.3 and 3.0 % (w/w) protein. 200 nmoles of each samples were loaded on an air-water interface in a Kimray-Greenfield surfactometer and successive compression-expansion cycles were applied. In the mixtures containing acidic phospholipids the presence of hydrophobic protein improved the maximum surface pressure (minimal surface tension) reached under compression and the stability of the monolayer. This fact suggested some interaction between protein and charged lipids, although no difference was noted between PG and PI. However, a difference was observed when the adsorption of DPPC/PG and DPPC/PI mixtures from the aqueous hypophase to the interface was assayed. Addition of hydrophobic protein improved the rate of adsorption of all the mixtures, it being more effective when the DPPC/PG or DPPC/PI ratio was 7:3. Mixtures with PI generally adsorbed faster than the mixtures with PG. The more rapid adsorption in the presence of PI could have been because lipid-protein interactions were favoured by the head group or the acyl chain composition of the PI in comparison to those of the PG. (Supported by the Medical Research Council of Canada, the Newfoundland Lung Association, and NATO).

# INTERACTION OF QUINOLONES WITH BILAYERS OF DPPC.

Pons M.[1], Estelrich J.[1], Montero T.[1], Freixas J.[2], Martorell C.[2], Hernández J.[1]
1.Unitat de Fisicoquímica, Facultat de Farmacia, U.B. 08028 Barcelona.Spain.
2.CENAVISA S.A. Laboratoris.43202 Reus (Spain)

Quinolones are a group of antibacterial agents with a mechanism of action based on the enzymes involved in the preparation of DNA for transcription. In vivo studies carried out with vesicles obtained by agitation of quinolones in phospholipids presence show better results than for those achieved with the free drug.This suggests that the study of the interaction between quinolones and DPPC could be helpful to understand the mechanism of interaction involved and further the encapsulation of these drugs in liposomes.

Quinolones were dried with DPPC at several molar ratios up to 50 % and after rehydratation with water and further extrussion, vesicle sizes werer monitored by quasi elastic light scattering for 10 days. Calculated enthalpies involved in the main transition were in all cases higher than for the pure phospholipid.These values ranged from 9.09 to 10.24 Kcal/mol but no linear relation between quinolone concentration and enthalpy was observed. In some cases several scans were necessary to get definitive endotherm indicating a low process of bilayer structure formation in quinolone presence. Transition temperatures also show minimum shifts towards lower values which could be related to the interaction with the polar groups of DPPC.

These findings are in agreement with variations in mean liposome diameter. Polydispersity values also suggest the formation of non-vesicular structures at high quinolone concentrations.

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**Deposition of biological lipids on solid planar substrates by Langmuir Blodgett technique**

Gertrud Puu, Inga Gustafsson, Per-Åke Ohlsson, Göran Olofsson and Åke Sellström, National Defence Research Establishment, Department of NBC Defence, S-901 82 Umeå, Sweden

With the goal to develop biosensors using membrane proteins as the sensing elements, we have investigated the influence of various factors on successful deposition of phospholipids by Langmuir Blodgett technique to form stable bilayers on planar supports.

We use factorial experimental design to screen factors of importance for transfer of the layer separately and for its stability. These experiments were performed on hydrophilic (glass, platinum, chromium) as well as on hydrophobic (silanized silicon wafers, alkylated gold) supports. The screening included factors such as temperature, pH, ionic composition of the subphase, deposition rate, pretreatment of the support and composition of the lipid mixture.

The last-mentioned parameter was also separately studied with a response surface design, in which we varied the proportions of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid (all as dipalmitoyl derivatives) and cholesterol, to achieve a stable first layer on platinum. In progress are experiments to find optimal conditions for the deposition of the second layer, thus creating a model membrane.

Our stepwise approach, i.e. to analyze each transferred layer separately, will hopefully result in a stable bilayer membrane into which membrane proteins can be reconstituted with retained activity.

# A NEW BILAYER MEMBRANE FORMING PHOSPHOLIPID ANALOGUE FOR PHOTOCOUPLING OF SOLUBLE PROTEINS

Saenger, M., Borle, F., Heller, M. and Sigrist, H.

Institute of Biochemistry, University of Berne,
CH-3012 BERNE

Photoactivable lipids N'-(1,2-diacyl-sn-glycero-3-phosphatidylethyl)-N-(m-trifluoromethyl-diazirine-phenyl))-thiourea (PED) have been applied for light-induced binding of soluble proteins to the outer surface of liposomes. PED-lipids combine the advantage of facile synthesis and timed carbene reactivity by photoactivation at wavelengths which are non-destructive to proteins. $C_{14}$-, $C_{16}$- and $C_{18}$-analogues have been prepared from synthetic phosphatidylethanolamines and 3-(trifluoro-methyl)-3-(m-isothiocyanophenyl)diazirine. Transition temperatures for $C_{14}$- and $C_{16}$-analogues were $10^0$ and $28^0$ respectively. In situ photoactivation of $C_{14}$-PED in planar bilayer membranes at wavelengths $\geq$ 320 nm did neither alter the mean conductivity nor the capacitance of the bilayer. Ionophore (valinomycin) and ion channel (gramicidin) activities were not impaired upon photoactivation. PED has been incorporated into liposomal membranes with varying amounts of non-photoactivable lipids. Light-dependent immobilization of proteins occured upon photoactivation of PED-containing liposomes in presence of soluble proteins. Therewith a promising new method for the formation of proteoliposomes is established.

# CHEMICAL CONTROLLED RELEASE OF ANALGESIC COMPOUNS FROM POLYMETHACRYLIC HYDROGELS

**J.San Román,[1] B.Levenfeld[1], C.Bunel[2] and J.P.Vairon[2]**
**1- Instituto de Ciencia y Tecnología de Polímeros**
**Juan de la Cierva 3, 28006-Madrid.**
**2- Lab.de Chimie Macromoleculaire,Université Pierre et Marie Curie**
**4,Place Jussieu-75252 Paris Cedex 05.**

In the last twenty years it has been observed an increasing attraction for the preparation of macromolecular systems known as polymeric drugs or polymer-bound drugs with clinical potential. In this sense, we have have prepared new polymers with pharmacological activity by means of the synthesis and free-radical polymerization of 4-(methacryloyloxy)acetanilide,**MOA**, an acrylic derivative of "**paracetamol**". Furthermore we have prepared hydrogels based on the copolymerization of this acrylic monomer,**MOA**, with 2-hydroxyethyl methacrylate,**HEMA**. The copolymers obtained can be used in the form of powder formulations or as active biomembranes with a clear hydrophilic character. The biocompatibility of polymeric formulations based on MOA has been tested "in vivo" and the use of poly(HEMA) is a guarantee of biocompatibility by the large experience of this kind of systems in surgery.

In this communication the heterogeneous hydrolytical behaviour of hydrogels prepared with different content of MOA is analysed by measuring the concentration of active metabolites released into the alkaline-buffered solution used as hydrolytical medium. Previously we have determined the swelling of hydrogels prepared with a MOA content between 1.0 and 5.0 wt-%, in order to known the diffusional character of the reagents and released species. The results obtained demonstrate that the swelling phenomenon is much faster than the hydrolytical chemical reaction. Thus the hydrolysis is studied on the basis of a kinetic scheme which considers the formation of different kind of residues, i.e., the sodium salt of 4-hydroxyacetanilide and 4-aminophenol, according to the UV spectra obtained at different times of treatment. The characteristic rate coefficients are evaluated from the experimental data on the basis of the suggested kinetic scheme. The results obtained make clear that a slow but continuous release of active metabolites from the hydrogel matrix is produced during at least twenty days of treatment in the buffered solution at 37°C.

# THE CALCIUM ANTAGONIST VERAPAMIL MODULATES THE INTERACTION OF ANTICANCER DRUGS WITH THE LIPID BILAYERS.

F. Soto, J. Canaves, J. Aleu, J.M. Gonzalez-Ros & J.A. Ferragut.

Department of Neurochemistry and Institute of Neurosciences. University of Alicante. 03080 Alicante. Spain.

The presence of the calcium antagonist verapamil (VRP) in artificial lipid vesicles, seems to modulate the effects that the anticancer drug daunomycin (DNM) induces in the lipid bilayer of the vesicles. This conclusion arises from equilibrium binding, fluorescence depolarization and differential scanning calorimetry (DSC) experiments using dimyristoylphosphatidylcholine (DMPC) or dipalmitoylphosphatidylcholine (DPPC) liposomes containing different DNM/VRP molar ratios. Thus, binding data analysis from Klotz plots indicate that the overall binding constant of DNM to the liposomes, decreases in the presence of VRP. Such effects are mainly due to a significant reduction in the apparent affinity constant, since the stoichiometry of binding seems not to be affected. On the other hand, the "fluidizing" effect of DNM below the phase transition of DPPC liposomes (measured by 1, 6 diphenylhexatriene (DPH) depolarization), can also be antagonized by the presence of VRP. The influence of VRP on the interaction of DNM with the liposomes is better visualized by DSC experiments, which describe how the changes produced by DNM in the thermotropic behavior of the liposomes, can be prevented by the calcium antagonist. Binding data of DNM to liposomes reveal that, at the VRP/DNM molar ratio used in DSC experiments, the effect of VRP can not be adscribed to alterations in the binding parameters.

These observations suggest that the reversal of multidrug resistance by calcium antagonists, in addition to being base on competition with the antineoplastics for binding to P-glycoproteins, could also involve modulation of the effects of the antitumor drugs at a plasma membrane level.

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# ON THE EFFECT OF LYSOPHOSPHATIDYLCHOLINE, PLATELET ACTIVATING FACTOR AND OTHER SURFACTANTS ON CALCIUM PERMEABILITY IN SARCOPLASMIC RETICULUM VESICLES

José A. Teruel, Fernando Soler and Juan C. Gómez-Fernández

Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, E-30071 Murcia, Spain

The effect of low concentrations of lysophosphatidylcholine (LPC), platelet-activating factor (PAF) and other surfactants (Triton X-100, $C_{12}E_8$, sodium dodecyl sulfate, sodium cholate, and sodium deoxycholate) on membrane permeability of native sarcoplasmic reticulum vesicles and sarcoplasmic reticulum lipid vesicles, has been studied. Triton X-100, $C_{12}E_8$, sodium dodecyl sulfate, sodium cholate and sodium deoxycholate were all able of permeabilizing membranes at concentrations of surfactants below their critical micellar concentration (c.m.c.) in both lipid and native vesicles, being the $K_{0.5}$ of calcium release from native vesicles lower than that from lipid vesicles. The values of these $K_{0.5}$ were well correlated with the corresponding c.m.c. values for each type of membrane.

However both LPC and PAF behaved in a different way since, although they induced permeabilization of the native vesicles at values of $K_{0.5}$ close to their c.m.c., their $K_{0.5}$ values for permeabilizing vesicles, prepared by using lipids extracted from sarcoplasmic reticulum, were much higher than their corresponding c.m.c.

This work was supported by grant No. PB87-0704 from DGICYT, Spain.

# THE BINDING PARAMETERS OF A HYDROPHOBIC CATIONIC DRUG TO PHOSPHOLIPID MEMBRANES.

Peter G. Thomas and Joachim Seelig

Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Utrecht, The Netherlands

Dept. of Biophysical Chemistry, Biozentrum, University of Basel, Basel, Switzerland

The interaction of cationic amphiphilic drugs with membranes has long been of interest to the membrane biochemist because of the pharmacological activity of such molecules. This activity usually takes place at the plasma membrane level and often involves an initial interaction of drug with the membrane phospholipids. This is reinforced by the amphiphilic nature of these drugs. It is generally thought that these drugs, when charged, are intercalated into a phospholipid bilayer with their hydrophobic moieties inserted into the acyl chain region and the charged groups positioned around the glycerol backbone/phosphate region, i.e. the interface. Owing to the greatly differing chemical structures of this class of molecule it is inevitable that they have quite different physicochemical properties. Unlike many of this group the calcium antagonist flunarizine is very hydrophobic even in its charged state. The binding of this drug to phosphatidylcholine bilayers has been investigated using centrifugation assays, zeta potential measurements and reaction microcalorimetry. The results reveal a very high binding coefficient with binding characteristics that can be explained by the Gouy-Chapman theory. Interestingly there is quite a strong exothermic component in the binding of this molecule to phospholipids which cannot simply be explained by the 'hydrophobic effect'

# MOLECULAR ORDER AND FLUIDITY OF THE PLASMA MEMBRANE OF HUMAN PLATELETS FROM TIME RESOLVED FLUORESCENCE DEPOLARIZATION.

M.Vélez, C.R. Mateo, Ma.P.Lillo, J.González and A.U.Acuña.
Instituto de Química-Física "Rocasolano", C.S.I.C. Serrano 119, 28006 Madrid, Spain.

Our interest is to study the role of the plasma membrane lipids in the regulation of platelet aggregation. It is known that the platelet aggregation process is sensitive to the cholesterol/phospholipid ratio, and to the phospholipid and fatty acid composition of the plasma membrane. The molecular mechanism by which the membrane lipids affect protein and overall cell function is not known. Our approach has been to, first, describe the lipid matrix physical parameters, molecular order and fluidity, of the native membrane of inactive intact platelets, of membrane fragments and of lipids extracted from that membrane, and then to observe the changes induced in these physical parameters by modifications in the lipid composition.

We determined the values of order parameters and apparent viscosities from the time-resolved fluorescence depolarization of the membrane probe 1,6 diphenyl 1,3,5 hexatriene (DPH) and some of its derivatives in the temperature range from 25 to 40°C. The experiments also showed that, for temperatures close to the physiological range, the fluorescence anisotropy of the probes gives indication of some sort of lipid segregation in the lipid bilayer. The cholesterol/phospholipid ratio in the membrane was then altered by incubating the platelets with cholesterol rich liposomes. The cholesterol exchange was verified with a detailed biochemical analysis of the membrane lipid species composition done by thin layer chromatography . The physical parameters of the three membrane preparations were assessed again.

# FORMATION AND GROWTH OF UNILAMELLAR VESICLES ACCORDING TO THE *THEORY OF THE VESICULAR GERM FORMATION*

A.O.Vila, C.Llácer, M.J.Martos, J.E.Figueruelo and F.J.Molina.
Unitat d'Investigació de Coloides, Dpt Química Física. Universitat de Valencia.
Avda Blasco Ibáñez 13, 46010 Valencia. Spain.

In this abstract we describe the first theorical results obtained by development of the theory of the Vesicular Germ Formation (TFGV) wich explain the formation process of unilamellar lipidical vesicles constitued by amphiphilic molecules.

For that, The TFGV establishes the balance between the necessary energy for liposome formation and the available energy of system for it. The surface energy and the energy asociated at the Laplace's overpressure are conected with the necessary energy while the chemical potential is conected with the available energy.

Tanford's (1), Ruckenstein's (2) and Mukerjee's (3) works are the reference for to establish the condition of the chemical equilibrium, then we are able to establish the critical metastable state for the formation of liposomes.

In its methodology the theory of the Vesicular Germ Formation introduce two novel concepts: one is the "equivalent radius" ($\rho$), defined for the expression: $\rho = r/e_0$ where r is the external radius of the vesicle and $e_0$ is the thickness bilayer and one is the "energetic balance" ($\delta$), defined for the expression : $\delta = \Delta\mu' \cdot e_0 / v_m \cdot \sigma$ where $\Delta\mu'$ is the excess of the chemical potential, wich stablishes the penetration degree in the system metastability, $v_m$ is the molar volume of the amphiphilic molecules and $\sigma$ is the surface tension of medium. The energetic balance stablishes the balance between the available energy for system and the mechanical energy of liposome.

So that the "energetic barrier" is defined as:

$$\varepsilon(\rho,\delta) = (5/3)\ (2\rho^2 - 2\rho + 1) - \delta \cdot (\rho^2 - \rho + 1/3)$$

The energetic balance data show the capacity of system for the formation of liposomes, also provide information about the aggregate geometry. When $\delta$ value is contained between 10/3 and 5 the formation of liposomes is spontaneous. The natural tendency of system to the chemical equilibrium ( $\delta$=0) involve the growth of vesicle to larger $\rho$ values. The infinite $\rho$ value involve the $\delta$ value has tendency towards 10/3. The experimental relationship of the theory of the Vesicular Germ Formation is carried out through the critical conditions when the energetic barrier is null, then we are named "*germs*" to the theorycal structures formed to establish its evolution capacity to the chemical equilibrium from metastable state.

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# STERIC IMMOBILIZATION OF LIPOSOMES AND PROTEOLIPOSOMES IN GEL BEADS BY FREEZE-THAWING AND BY FREEZE-DRYING AND REHYDRATION PROCEDURES

Q. Yang* and P. Lundahl, Department of Biochemistry, Biomedical Center, University of Uppsala, Uppsala, Sweden

Immobilized liposomes and proteoliposomes in gel beads have been used in our group as stationary phases for aqueous column chromatography, for studies on biomembrane components incorporated in the lipid bilayers. We have recently developed another method for liposome immobilization which is based on fusion of small liposomes in gel beads upon freeze-thawing and upon freeze-drying and rehydration procedures.

Egg yolk phospholipids (EYP) liposomes and proteoliposomes were prepared by detergent removal from 0.1 M EYP solution on a Sephadex G-50 column. The liposome suspension was concentrated in a Minicon B concentrator (Amicon). 0.55 or 1.1 ml of the concentrated liposome suspension was well mixed with 0.7 g of suction-dried gel or 76 mg of freeze-dried Sephacryl S-1000 gel in a conical-bottom tube, respectively. The mixture was then either frozen in the tube in dry-ice/ethanol and thawed after 10 min, or freeze-dried overnight and rehydrated. Non-immobilized liposomes were removed by centrifugation.

By mixing of suction-dried gel with a liposome suspension for freeze-thawing we achieved an immobilization capacity of 25-80 µmol of phospholipids per ml gel at initial liposome concentration of 100-300 mM, an increase by a factor of 3-8 compared with the capacity obtained by dialysis method reported previously. Similar capacities were obtained for proteo-liposomes. A slightly higher capacity, 20-100 µmol of phospholipids per ml gel, could be obtained at relatively low initial liposome concentration of 50-150 mM by use of freeze-dried gel, since the dried gel caused little dilution of the liposome suspension. Immobilization of liposomes by freeze-drying and rehydration resulted in capacities of 90-110 µmol per ml gel at initial liposome concentrations of 52, 105 and 210 mM with the same amounts of phospholipids, 230 µmol. This result shows a much lower effect of the initial liposome concentration on the immobilization capacity obtained by freeze-drying and rehydration than that obtained by freeze-thawing. 17 mg of lysozyme could bind to 1 ml packed Sephacryl S-1000 gel with immobilized phosphatidylserine liposomes (38 µmol of phospholipids). This high capacity for protein binding makes immobilized liposomes more interesting for chromatographic purposes.

# DIFFERENT UNSATURATED PHOSPHATIDYLCHOLINES ARE AFFECTED TO VARIOUS EXTENTS BY CHOLESTEROL

J. Hernandez-Borrell[1] and K.M.W. Keough [1,2]

Department of Biochemistry[1] and Discipline of Pediatrics[2], Memorial University of Newfoundland, St. John's, Newfoundland, Canada, A1B 3X9

Cholesterol has long been known to influence the gel to liquid-crystalline (l.c.) phase transitions of phospholipids in bilayer membranes. We have studied the effect of cholesterol on the gel to l.c. transitions of a series of unsaturated phosphatidylcholines (PC) commonly found in biological membranes (16:0-18:1 PC, 16:0-18:2 PC, 16:0-20:4 PC and 16:0-22:6 PC) and a saturated analogue (16:0-18:0 PC). Membranes containing these lipids plus varying amounts of cholesterol were investigated by differential scanning calorimetry. For the pure PC neither the transition temperatures or the enthalpy changes of the transitions were directly related to the number of double bonds in the sn-2 chain. The amount of cholesterol required to remove the calorimetrically observed transition was different for each of the lipids studied. The transition of 16:0-18:2 PC was abolished by the lowest amount of cholesterol (<17 mol%); transitions of 16:0-18:1 PC and 16:0-20:4 PC were similarly affected, their transitions being removed by 20-25 mol% cholesterol; 16:0-18:0 PC and 16:0-22:6 PC were also similarly influenced by cholesterol, their transitions being removed by about 35 mol% sterol. These results suggest that while most phosphatidylcholine-cholesterol interactions may be qualitatively similar, quantitative aspects of various lipid-cholesterol systems may be quite different from one another. It is of significance to the understanding of roles of cholesterol in natural membranes that lipids with different degrees of unsaturation can be influenced to considerably different extents by the presence of a given amount of cholesterol. (Supported by the Medical Research Council of Canada).

# A study of polymer-lipid interactions monolayer

NUÑEZ RECUERO, J.L.**; BALLESTEROS PAPANTONAKIS, M.P.**; LASTRES GARCIA, J.L.**; CASTRO RUIZ, R.M.*

*Departamento de Química Física Farmacéutica.
**Departamento de Farmacia y Tecnología Farmacéutica.
Facultad de Farmacia. Universidad Complutense. 28040. MADRID.

Polymers are often used as drug carriers, specially for the controlled release of drugs. Therefore the study of their interactions with biological materials is an actual and promissing field in Pharmacy. We have studied with a surface balance the interaction of poly(methyl vinyl ether/maleic anhydride) (PVM/MA) with a membrane model, the L-α-dimyristoil phosphatidylcholine (DMPC).

We started forming DMPC monolayers, by extension of a lipid solution in methanol on several buffer systems. After the solvent was evaporated the surface pressure vs area curves were measured. A polymer solution was extended in the same conditions and studied in a similar way, cheking that a polymer monolayer was formed in spite of the polymer solubility in water. Later on DMPC-PVM/MA mixtures were studied in the same experimental conditions (substrate, buffer, pH, temperature and so on).

The bidimensional diagrams (molecular area at a given constant pressure vs mixture composition) show that there are atractive forces between DMPC and the polymer in the mixture monolayers. These results indicate that this polymer could be used as a bioadhesive substance in pharmaceutical dosage forms.

# LIST OF PARTICIPANTS

ALONSO, Alicia; Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad del País Vasco, 48080 Bilbao, Spain

ALVING, Carl R.; Department of Membrane Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100. USA

ARANDA, Francisco J.; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30071, Murcia, Spain

BRACO, Lorenzo; Depto. Bioquímica y Biología Molecular, Universidad de Valencia, Burjassot, E-46100, VALENCIA, Spain

BRON, Romke; Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands

CASALS, Cristina; Depto. Bioquímica, Facultad de CC.Químicas, Universidad Complutense, 28040 Madrid, Spain

CHAKRABARTI, Ajoy;, Department of Biochemistry, 2146 Health Sciences Mail, University of British Columbia, Vancouver, B.C.Canada, V6T 1W5

CHAPMAN, Dennis (FRS); University of London, Department of Protein and Molecular Biology, Royal Free Hospital School of Medicine Rowland Hill Street, London NW3 2PF. UK

CORBALAN, M. Senena; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30071, Murcia, Spain

CORTIJO, Manuel; Departamento de Química Física, Facultad de Farmacia, Universidad Complutense, 28040, Madrid, Spain

DE MADARIAGA, M. Africa; Depto. de Bioquímica y Fisiología, Facultad de Químicas, Universidad de Barcelona, Martí i Franquès 1, 08020 Barcelona, Spain

DUNCAN, Ruth; Cancer Research Campaing Polymer, Controlled Drug Delivery Research Group, Dept. of Biological Sciences, University of Keele, Keele, Staffs, ST5 5BG, U.K.

EGEA GRAS, M. Antonia; Depto. de Farmacia, Unidad de Fisicoquímica, Facultad de Farmacia, Avda. Diagonal s/n, 08020 Barcelona, Spain

ELORZA BARROETA, Begoña; Departamento de Química Física, Facultad de Farmacia, Universidad Complutense, 28040, Madrid, Spain

FERNANDEZ BALLESTER, Gregorio; Departamento de Neuroquímica, Instituto de Neurociencias, Universidad de Alicante, 03080 ALICANTE, Spain

FERNANDEZ-BELDA, Francisco; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30071, Murcia, Spain

FERNANDEZ SALGUERO, Pedro; Depto. Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Extremadura, 06080 Badajoz, Spain

FERRAGUT, José A.; Departamento de Neuroquímica, Instituto de Neurociencias, Universidad de Alicante, 03080 ALICANTE, Spain

FONSECA CHACHARO, M. José; Laboratorio de Péptidos, Consejo Superior de Investigaciones Científicas, Centro de Investigación y Desarrollo, Jordi Girona, 18-26, 08034 Barcelona, Spain

GABER, Bruce; Naval Research Laboratory, 4555 Overlook Avenue, Washington, DC 20375-5000. USA

GIAMMARA, Beverly; Analytical Electron Microscopy Laboratory, Graduate Programs and Research, University of Louisville, Louisville, Kentucky 40292, U.S.A.

GOMEZ-FERNANDEZ, Juan C.; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, Espinardo, 30071, Murcia. Spain

GONZALEZ-ROS, José M.; Departamento de Neuroquímica, Facultad de Medicina. Universidad de Alicante, 03080 Alicante. Spain

GOÑI, Félix M.; Departamento de Bioquímica, Facultad de Ciencias, Universidad del País Vasco, 48080 Bilbao. Spain

GRAINGER, David; Dept. of Chemical & Biological Sciences, Oregon Graduate Center, 19699 N.W. Von Neumann Drive, Beaverton, OR 97006-1999, U.S.A.

GUTIERREZ MERINO, Carlos; Depto. Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Extremadura, 06080 Badajoz, Spain

HANKER, Jacob S.; Biomedical Engineering Curriculum, University of North Carolina, Chapel Hill, NC 27599, USA

HAUSER, Helmut; Laboratorium für Biochemie, Eidgenössische Technische Hochschule, CH-8092-Zürich, Switzerland

HERNANDEZ-BORRELL, Jordi; Unidad de Fisicoquímica, Departamento de Farmacia, Universidad de Barcelona, 08028 Barcelona, Spain

KEOUGH, Kevin M. W.; Dept. Biochemistry, Memorial University of Newfoundland, St. John's. Newfoundland, Canada A1B 3X9

KILLIAN, Antoinette; State University of Utrecht, Institute of Molecular Biology and Medical Biotechnology, Padualaan 8, 3584 CH Utrecht. The Netherlands

LESERMAN, Lee; Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille CEDEX 9, France

LOPEZ, Francisco; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30071, Murcia, Spain

LLACER ALMELA, Carmen; Unidad de Investigación de Coloides, Dept. Química Física, Universidad de Valencia, Avda. Blasco Ibáñez 13, 46010 VALENCIA, Spain

LLOVET LOMBARTE, M. Isabel; Depto. Farmacia. Unidad de Fisicoquímica, Facultad de Farmacia, Avda. Diagonal s/n, 08020 Barcelona, Spain

MARTINEZ, Francisco; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30071, Murcia, Spain

MARTOS GUILLEN, M. José; Unidad de Investigación de Coloides, pt. Química Física, Universidad de Valencia, Avda. Blasco Ibáñez13, 46010 VALENCIA, Spain

MOLINA LUCAS, Francisco J.; Dpto. Química Física, Facultad de Farmacia, Universidad de Valencia, Avda. Blasco Ibáñez 13, 46010 Valencia, Spain

MONTE, Giovanni; Clinical Pathology Unit, Dept. of Ethiology/Pathophysiology, NATIONAL CARDIOVASCULAR CENTER, 5 - 7-I Fujishiro-dai, Suita,, Osaka, 565, Japan

MORA, Margarita; Depto. de Bioquímica y Fisiología, Facultad de Químicas, Universidad de Barcelona, Martí i Franquès 1, 08020 Barcelona, Spain

NICOLAU, Claude; IBT Center for Protein Engineering, Membrane Biotechnology Section, TAMU Research Park, 100 Research Parkway, Suite 150, College Station, Texas 77840, USA

NUÑEZ RECUERO, José L.; Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain

ORTIZ, Antonio; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30071, Murcia, Spain

PACKER, Lester; University of California, Department of Molecular and Cell Biology, 251, Life Science Addition, Membrane Bioenergetics Group, Berkeley, California. 94720, USA

PEREZ GIL, Jesus; Depto. Bioquímica, Facultad de CC.Químicas, Universidad Complutense, 28040 Madrid, Spain

PONS, Miquel; Depto. Física Química, Facultad de Farmacia, Avda. Diagonal s/n, 08028 BARCELONA, Spain

PRIETO, Manuel J.E.; Centro de Química Física Molecular da Universidade Técnica de Lisboa (INIC), Complexo I, IST, Avda. Rovisco Pais, 1096 Lisboa Codex, PORTUGAL

PUU, Gertrud; Swedish Defence Research Establishment, Department of NBC Defence, S-901 82 UMEA, Sweden

QUINN, Peter J.; Kings College London, Department of Biochemistry, Campden Hill Road, Kensington, London W87 AH. UK

RODRIGUEZ-VALERA, Francisco; Departamento de Microbiología, Facultad de Medicina, Universidad de Alicante, Apartado 374, 03080-Alicante. Spain

ROSELL, Francisco; Depto. de Bioquímica y Fisiología, Facultad de Química, Universidad de Barcelona, Martí i Franquès 1, 08028 Barcelona, Spain

RUDOLPH, Alan; Department of the Navy, Naval Research Laboratory, Washington, D.C. 20375-5000, U.S.A.

RUIZ SANZ, Javier; Depto. Química Física, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain

SAENGER, Michael; Institute of Biochemistry, University of Berne, Freiestr. 3, CH- 3012 BERNE, Switzerland

SAINZ VILLANUEVA, Cristina; Departamento de Química Física, Facultad de Farmacia, Universidad Complutense, 28040, Madrid, Spain

SALGADO, Josefa; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30071, Murcia, Spain

SAN ROMAN, Julio; Instituto de Ciencia y Tecnología de, Polímeros, CSIC, c/ Juan de La Cierva 3, 28006- MADRID, Spain

SANCHEZ-MIGALLON, M. Paz; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30071, Murcia, Spain

SEIDERS, Reginald P.; Chief, Chemistry & Biological Science Branch, European Research Office, U.S. Army, 223-231 Old Marylebone Road, London, NW1, England

SHIPLEY, Graham G.; Department of Biophyscs, Boston University School of Medicine, 80 East Concord St., Boston, MA 02118, U.S.A.

SOTO LUCAS, Florentina; Departamento de Neuroquímica, Instituto de Neurociencias, Universidad de Alicante, 03080 ALICANTE, Spain

STORM, G.; Department of Pharmaceutics, Faculty of Pharmacy, University of Utrecht, PO Box 80.082, NL- 3508 TB Utrecht, The Netherlands

TERUEL, José A.; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30071, Murcia, Spain

THOMAS, Peter G.; Institute of Molecular Biology and, Medical Biotechnology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

THOMPSON, Nancy E.; Department of Chemistry, CB 3290, Venable Hall, University of North Carolina, Chapel Hill, North Carolina 27599, USA

VALDES, James J.; Scientific Advisor for Biotechnology, U.S. Army Chemical Research, Development and, Engineering Center, Attention: SMCCR-TDB, Aberdeen Proving Ground, Maryland 21010 - 5423, U.S.A.

VELEZ, Marisela; Instituto de Química Física "Rocasolano", Consejo Superior de Investigaciones Científicas, Serrano 119, 28006 Madrid, Spain

VILA BUSO, Amparo; Dpto. Química Física, Facultad de Farmacia, Universidad de Valencia, Avda. Blasco Ibáñez 13, 46010 Valencia, Spain

VILLALAIN, José; Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30071, Murcia, Spain

WILSCHUT, Jan; University of Groningen, Laboratory of Phisiological Chemistry, Bloemsingel 10, 9712 KZ, Groningen, The Netherlands

WIRTZ, Karel W.A.; State University of Utrecht, Centre for Biomembranes and Lipid Enzymology, P.O. BOX 80054 , 3508 TB Utrecht, The Netherlands

WOLF, Claude; Saint Antoine Université Paris VI, U.R.A. C.N.R.S. 1243 C.H.U., 27, rue Chaligny 75012, Paris. France.

YANG, Quing; Department of Biochemistry, Biomedical Center, University of Upsala, P.O. Box 576, S-75123 Upsala, Sweden

# AUTHOR INDEX

(Numbers in parenthesis refer to the corresponding Abstract of Lecture "AL" or Abstract of Poster "AP")

| | |
|---|---|
| LLOVET LOMBARTE, M. Isabel | (AP14) |
| MARTINEZ, Francisco | |
| MARTOS GUILLEN, M. José | (AP15) |
| MOLINA LUCAS, Francisco J. | (AP16) |
| MONTE, Giovanni | (AP17) |
| MORA, Margarita | (AP8) |
| NICOLAU, Claude | (AL12) |
| NUÑEZ RECUERO, José L. | (AP30) |
| ORTIZ, Antonio | (AP2, AP4) |
| PACKER, Lester | (AL6) |
| PEREZ GIL, Jesus | (AP18) |
| PONS, Miquel | (AP19) |
| PRIETO, Manuel J.E. | (AP5) |
| PUU, Gertrud | (AP20) |
| QUINN, Peter J. | (AL3) |
| RODRIGUEZ-VALERA, Francisco | (AL22) |
| ROSELL, Francisco | (AP8) |
| RUDOLPH, Alan | (AL21) |
| RUIZ SANZ, Javier | (AP7) |
| SAENGER, Michael | (AP21) |
| SAINZ VILLANUEVA, Cristina | |
| SALGADO, Josefa | |
| SAN ROMAN, Julio | (AP22) |
| SANCHEZ-MIGALLON, M. Paz | |
| SEIDERS, Reginald P. | |
| SHIPLEY, Graham G. | (AL18) |
| SOTO LUCAS, Florentina | (AP23) |
| STORM, G. | (AL15) |
| TERUEL, José A. | (AP24) |
| THOMAS, Peter G. | (AP25) |
| THOMPSON, Nancy E. | (AL17) |
| VALDES, James J. | |
| VELEZ, Marisela | (AP26) |
| VILA BUSO, Amparo, O. | (AP27) |
| VILLALAIN, José | |
| WILSCHUT, Jan | (AL11) |
| WIRTZ, Karel W.A. | (AL7) |
| WOLF, Claude | (AL4) |
| YANG, Quing | (AP28) |